



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(54) Title:</b> YEAST STRAINS AND MODIFIED ALBUMINS  <b>(57) Abstract</b> <p>Albumin, for example human albumin, is expressed and secreted in yeast which has been mutated to lack the yeast aspartyl protease 3 (Yap3p) or its equivalent, thereby reducing the production of a 45kD albumin fragment. A further reduction is achieved by additionally deleting the Kex2p function. Alternatively, a modified albumin is prepared which is not susceptible to Yap3p cleavage, for example human albumin which is R410A, K413Q and K414Q.</p> <div data-bbox="832 1545 1688 2439"><table border="0"><thead><tr><th>A</th><th>B</th><th>C</th><th></th></tr></thead><tbody><tr><td colspan="3"></td><td></td></tr><tr><td></td><td></td><td></td><td>--rHA monomer</td></tr><tr><td></td><td></td><td></td><td>--fragment</td></tr></tbody></table></div>			A	B	C									--rHA monomer				--fragment
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## YEAST STRAINS AND MODIFIED ALBUMINS

### Field of the invention

- 5 The present invention relates to the production of recombinant human albumin (rHA) by yeast species.

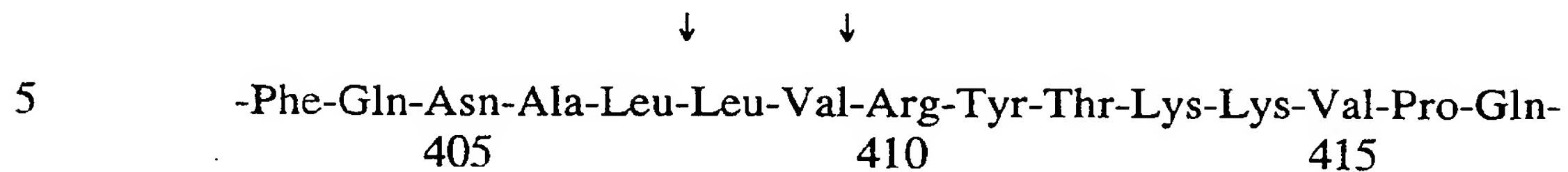
### Background and prior art

- 10 Human serum albumin (HSA) is a protein of 585 amino acids that is responsible for a significant proportion of the osmotic pressure of serum, and also functions as a carrier of endogenous and exogenous ligands. It is used clinically in the treatment of patients with severe burns, shock, or blood loss, and at present is produced commercially by extraction from human blood. The  
15 production of recombinant human albumin (rHA) in microorganisms has been disclosed in EP 330 451 and EP 361 991.

In recent years yeast species have been widely used as a host organisms for the production of heterologous proteins (reviewed by Romanos *et al*, 1992),  
20 including rHA (Sleep *et al*, 1990, 1991; Fleer *et al*, 1991). Yeasts are readily amenable to genetic manipulation, can be grown to high cell density on simple media, and as eukaryotes are suitable for production of secreted as well as cytosolic proteins.

- 25 When *S. cerevisiae* is utilised to produce rHA, the major secreted protein is mature 67kDa albumin. However, a 45kDa N-terminal fragment of rHA is also observed (Sleep *et al*, 1990). A similar fragment is obtained when rHA is expressed in *Kluyveromyces* sp. (Fleer *et al*, 1991) and *Pichia pastoris* (EP 510 693). The fragment has the same N-terminal amino acid sequence as  
30 mature rHA, but the carboxy terminus is heterogeneous and occurs between

Phe<sup>403</sup> and Val<sup>409</sup> with the most common termini being Leu<sup>407</sup> and Val<sup>409</sup> (Geisow *et al*, 1991), as shown below.



The amount of fragment produced, as a percentage of total rHA secreted, varies with both the strain and the secretion leader sequence utilised, but is never reduced to zero (Sleep *et al*, 1990). We have also found that the amount of fragment produced in high cell density fermentation (75-100g/L cell dry weight) is approximately five times higher than in shake flask cultures.

15 The 45kDa albumin fragment is not observed in serum-derived human serum albumin (HSA), and its presence as non-nature-identical material in the recombinant product is undesirable. The problem addressed by the present invention is to reduce the amount of the 45kDa fragment in the product. The simplest and most obvious approach would have been to have purified it away  
20 from the full length albumin, as proposed by Gist-brocades in EP 524 681 (see especially page 4, lines 17-22). However, we have chosen a different approach, namely to try to avoid its production in the first place.

25 Sleep *et al* (1990) postulated that rHA fragment is produced within the cell and is not the result of extra-cellular proteolysis. These authors codon-optimised the HSA cDNA from Glu<sup>382</sup> to Ser<sup>419</sup> but this had no effect on production of rHA fragment. They noted that a potential Kex2p processing site in the rHA amino acid sequence, Lys<sup>413</sup>Lys<sup>414</sup>, is in close proximity to the heterogeneous carboxy terminus of the fragment, but neither use of a *kex2* host strain (ie a strain harbouring a mutation in the *KEX2* gene such that it does not produce the Kex2p protease), nor removal of the potential cleavage site by site-directed

mutagenesis of the codon for Lys<sup>414</sup>, resulted in reduction in the amount of the fragment.

There is a vast array of yeast proteases which could, in principle, be degrading  
5 a desired protein product, including (in *S. cerevisiae*) yscA, yscB, yscY, yscS, other vacuolar proteinases, yscD, yscE, yscF (equivalent to kex2p), ysc $\alpha$ , yscIV, yscG, yscH, yscJ, yscE and kex1.

Bourbonnais *et al* (1991) described an *S. cerevisiae* endoprotease activity  
10 specific for monobasic sites, an example of which (Arg<sup>410</sup>) exists in this region of albumin. This activity was later found to be attributable to yeast aspartyl protease 3 (Yap3) (Bourbonnais *et al*, 1993), an enzyme which was originally described by Egel-Mitani *et al* (1990) as an endoprotease similar to Kex2p in specificity, in that it cleaved at paired basic residues. Further work suggested  
15 that Yap3p is able to cleave monobasic sites and between, and C-terminal to, pairs of basic residues, but that cleavage at both types of sites is dependent on the sequence context (Azaryan *et al*, 1993; Cawley *et al*, 1993).

As already discussed, the region of the C-terminus of rHA fragment contains  
20 both a monobasic (Arg<sup>410</sup>) and a dibasic site (Lys<sup>413</sup>Lys<sup>414</sup>). However, even though a Kex2p-like proteolytic activity is present in human cells and is responsible for cleavage of the pro sequence of HSA C-terminal to a pair of arginine residues, the fragment discussed above is not known to be produced in humans. This indicates that the basic residues Arg<sup>410</sup>, Lys<sup>413</sup> and Lys<sup>414</sup> are  
25 not recognised by this Kex2p-like protease, in turn suggesting that this region of the molecule may not be accessible to proteases in the secretory pathway. Thus, the Yap3p protease could not have been predicted to be responsible for the production of the 45kDa fragment. In addition, Egel-Mitani *et al* (1990 *Yeast* 6, 127-137) had shown Yap3p to be similar to Kex2p in cleaving the  
30 MF $\alpha$  propheromone. Since removal of the Kex2p function alone does not

reduce the amount of the fragment produced, there was no reason to suppose that removal of the Yap3p function would be beneficial. Indeed, Bourbonnais *et al* (1993) showed that *yap3* strains had a decreased ability to process pro-somatostatin, and therefore taught away from using *yap3* strains in the  
5 production of heterologous proteins.

### Summary of the invention

The solution to the problem identified above is, in accordance with the  
10 invention, to avoid or at least reduce production of the fragment in the initial fermentation, rather than to remove it during purification of the albumin. We have now found that, out of the 20 or more yeast proteases which are so far known to exist, it is in fact the Yap3p protease which is largely responsible for the 45kD fragment of rHA produced in yeast. The present invention provides  
15 a method for substantially reducing the amount of a 45kDa fragment produced when rHA is secreted from yeast species. The reduction in the amount of fragment both improves recovery of rHA during the purification process, and provides a higher quality of final product. A further, and completely unexpected, benefit of using *yap3* strains of yeast is that they can produce 30-  
20 50% more rHA than strains having the Yap3p function. This benefit cannot be accounted for merely by the reduction of rHA fragment from ~15% to 3-5%.

Thus, one aspect of the present invention provides a process for preparing albumin by secretion from a yeast genetically modified to produce and secrete  
25 the albumin, comprising culturing the yeast in a culture medium such that albumin is secreted into the medium, characterised in that the yeast cells have a reduced level of yeast aspartyl protease 3 proteolytic activity.

Preferably, the said proteolytic activity is an endoprotease activity specific for  
30 monobasic sites and for paired basic amino acids in a polypeptide.



Suitably, the yeast is *S. cerevisiae* which lacks a functional *YAP3* gene. However, the invention is not limited to the use of *S. cerevisiae*, since the problem of 45 kDa fragment production is found also in other yeast genera, for example *Pichia* and *Kluyveromyces*, which shows that they have equivalent proteases (ie Yap3p proteolytic activity); see Clerc *et al* (1994), page 253. We have confirmed this by hybridisation analysis to locate homologues of Yap3p in non-*Saccharomyces* genera. A gene is regarded as a homologue, in general, if the sequence of the translation product has greater than 50% sequence identity to Yap3p. In non-*Saccharomyces* genera, the Yap3p-like protease and its gene may be named differently, but this does not of course alter their essential nature.

The level of fragment can be reduced still further if, as well as substantially eliminating the Yap3p proteolytic activity, the Kex2p function is also substantially eliminated even though, as mentioned above, elimination of the Kex2p function alone does not affect the level of fragment. As in the case of Yap3p, the Kex2p function is not restricted to *Saccharomyces*; see Gellissen *et al* (1992), especially the sentence bridging pages 415 and 416, showing that *Pichia* has a Kex2p function. The genes encoding the Kex2p equivalent activity in *Kluyveromyces lactis* and *Yarrowia lipolytica* have been cloned (Tanguy-Rougeau *et al*, 1988; Enderlin & Ogrydziak, 1994).

A suitable means of eliminating the activity of a protease is to disrupt the host gene encoding the protease, thereby generating a non-reverting strain missing all or part of the gene for the protease (Rothstein, 1983). Alternatively, the activity can be reduced or eliminated by classical mutagenesis procedures or by the introduction of specific point mutations by the process of transplacement (Winston *et al*, 1983). Preferably, the activity of the enzyme is reduced to at most 50% of the wild-type level, more preferably no more than 25%, 10% or 5%, and most preferably is undetectable. The level of Yap3p proteolytic

activity may be measured by determining the production of the 45 kDa fragment, or by the  $^{125}\text{I}$ - $\beta_{\text{h}}$ -lipoprotein assay of Azaryan *et al* (1993), also used by Cawley *et al* (1993). Kex2p proteolytic activity may similarly be measured by known assays, for example as set out in Fuller *et al* (1989).

5

The albumin may be a human albumin, or a variant thereof, or albumin from any other animal.

By "variants" we include insertions, deletions and substitutions, either  
10 conservative or non-conservative, where such changes do not substantially alter the oncotic, useful ligand-binding or non-immunogenic properties of albumin. In particular, we include naturally-occurring polymorphic variants of human albumin; fragments of human albumin which include the region cleaved by Yap3p, for example those fragments disclosed in EP 322 094 (namely HSA (1-  
15 n), where n is 369 to 419) which are sufficiently long to include the Yap3p-cleaved region (ie where n is 403 to 419); and fusions of albumin (or Yap3p-cleavable portions thereof) with other proteins, for example the kind disclosed in WO 90/13653.

20 By "conservative substitutions" is intended swaps within groups such as Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr.

Such variants may be made using the methods of protein engineering and site-directed mutagenesis as described below.

25

A second aspect of the invention provides a modified albumin having at least 90% sequence identity to a naturally-occurring albumin, which naturally-occurring albumin is susceptible to cleavage with the *S. cerevisiae* yeast aspartyl protease 3 (Yap3p) when expressed in yeast, characterised in that the  
30 modified albumin is not susceptible to such cleavage.



Preferably, the modified albumin lacks a monobasic amino acid present in the naturally-occurring albumin protein. Suitably, the said monobasic amino acid is arginine. Conveniently, the modified albumin additionally lacks a pair of basic amino acids present in the naturally-occurring albumin, especially any of  
5 Lys, Lys; Lys, Arg; Arg, Lys; or Arg, Arg. Thus, in one particular embodiment, the naturally-occurring albumin is human albumin and the modified protein lacks Arg<sup>410</sup> and, optionally, one or both Lys<sup>413</sup>Lys<sup>414</sup> lysines. For example, the modified albumin may be human albumin having the amino acid changes R410A, K413Q, K414Q. Equivalent modifications in bovine  
10 serum albumin include replacing the Arg<sup>408</sup> and/or one or both of Arg<sup>411</sup>Lys<sup>412</sup>. The person skilled in the art will be able to identify monobasic sites and pairs of basic residues in other albumins without difficulty.

The numbering of the residues corresponds to the sequence of normal mature  
15 human albumin. If the albumin is a variant (for example a polymorphic form) having a net deletion or addition of residues N-terminal to the position identified, then the numbering refers to the residues of the variant albumin which are aligned with the numbered positions of normal albumin when the two sequences are so aligned as to maximise the apparent homology.

20

A third aspect of the invention provides a polynucleotide encoding such a modified albumin.

The DNA is expressed in a suitable yeast (either the DNA being for a modified  
25 albumin, or the yeast lacking the Yap3p function) to produce an albumin. Thus, the DNA encoding the albumin may be used in accordance with known techniques, appropriately modified in view of the teachings contained herein, to construct an expression vector, which is then used to transform an appropriate yeast cell for the expression and production of the albumin.

30

The DNA encoding the albumin may be joined to a wide variety of other DNA sequences for introduction into an appropriate host. The companion DNA will depend upon the nature of the host, the manner of the introduction of the DNA into the host, and whether episomal maintenance or integration is desired.

5

Generally, the DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. The vector is then introduced into the host through standard techniques and, generally, it will be necessary to select for transformed host cells.

10

Host cells that have been transformed by the recombinant DNA of the invention are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the expression and secretion of the albumin, which can then be recovered, as is known.

15

Useful yeast plasmid vectors are pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (YIps) and incorporate the yeast selectable markers *HIS3*, *TRP1*, *LEU2* and *URA3*. Plasmids pRS413-416 are Yeast Centromere plasmids (YCps). Other yeast expression plasmids are disclosed in EP-A-258 067, EP-A-286 424 and EP-A-424 117.

20

25 The polynucleotide coding sequences encoding the modified albumin of the invention may have additional differences to those required to produce the modified albumin. For example, different codons can be substituted which code for the same amino acid(s) as the original codons. Alternatively, the substitute codons may code for a different amino acid that will not affect the activity or immunogenicity of the albumin or which may improve its activity

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or immunogenicity, as well as reducing its susceptibility to a Yap3p protease activity. For example, site-directed mutagenesis or other techniques can be employed to create single or multiple mutations, such as replacements, insertions, deletions, and transpositions, as described in Botstein and Shortle  
5 (1985). Since such modified coding sequences can be obtained by the application of known techniques to the teachings contained herein, such modified coding sequences are within the scope of the claimed invention.

Exemplary genera of yeast contemplated to be useful in the practice of the  
10 present invention are *Pichia*, *Saccharomyces*, *Kluyveromyces*, *Candida*, *Torulopsis*, *Hansenula* (now reclassified as *Pichia*), *Histoplasma*, *Schizosaccharomyces*, *Citeromyces*, *Pachysolen*, *Debaromyces*, *Metschnikowia*, *Rhodospiridium*, *Leucosporidium*, *Botryosphaeria*, *Sporidiobolus*, *Endomycopsis*, and the like. Preferred genera are those selected from the group consisting of  
15 *Pichia*, *Saccharomyces*, *Kluyveromyces*, *Yarrowia* and *Hansenula*. Examples of *Saccharomyces* sp. are *S. cerevisiae*, *S. italicus* and *S. rouxii*. Examples of *Kluyveromyces* sp. are *K. fragilis* and *K. lactis*. Examples of *Hansenula* (*Pichia*) sp. are *H. polymorpha* (now *Pichia angusta*), *H. anomala* (now *P. anomala*) and *P. pastoris*. *Y. lipolytica* is an example of a suitable *Yarrowia*  
20 species.

Methods for the transformation of *S. cerevisiae* are taught generally in EP 251 744, EP 258 067 and WO 90/01063, all of which are incorporated herein by reference. Suitable promoters for *S. cerevisiae* include those associated with  
25 the *PGK1* gene, *GAL1* or *GAL10* genes, *CYC1*, *PHO5*, *TRP1*, *ADH1*, *ADH2*, the genes for glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, triose phosphate isomerase, phosphoglucose isomerase, glucokinase,  $\alpha$ -mating factor pheromone, a-mating factor pheromone, the *PRB1* promoter, the *GPD1* promoter, and hybrid  
30 promoters involving hybrids of parts of 5' regulatory regions with parts of 5'

regulatory regions of other promoters or with upstream activation sites (eg the promoter of EP-A-258 067).

Convenient regulatable promoters for use in *Schizosaccharomyces pombe* are  
5 the thiamine-repressible promoter from the *nmt* gene as described by Maundrell (1990) and the glucose-repressible *fbp1* gene promoter as described by Hoffman & Winston (1990).

Methods of transforming *Pichia* for expression of foreign genes are taught in,  
10 for example, Cregg *et al* (1993), and various Phillips patents (eg US 4 857 467, incorporated herein by reference), and *Pichia* expression kits are commercially available from Invitrogen BV, Leek, Netherlands, and Invitrogen Corp., San Diego, California. Suitable promoters include *AOX1* and *AOX2*.

15 The Gellissen *et al* (1992) paper mentioned above and Gleeson *et al* (1986) *J. Gen. Microbiol.* 132, 3459-3465 include information on *Hansenula* vectors and transformation, suitable promoters being *MOX1* and *FMD1*; whilst EP 361 991, Fleer *et al* (1991) and other publications from Rhône-Poulenc Rorer teach how to express foreign proteins in *Kluyveromyces* spp., a suitable promoter being  
20 *PGK1*.

The transcription termination signal is preferably the 3' flanking sequence of a eukaryotic gene which contains proper signals for transcription termination and polyadenylation. Suitable 3' flanking sequences may, for example, be  
25 those of the gene naturally linked to the expression control sequence used, ie may correspond to the promoter. Alternatively, they may be different in which case the termination signal of the *S. cerevisiae ADH1* gene is preferred.

The albumin is initially expressed with a secretion leader sequence, which may  
30 be any leader effective in the yeast chosen. Leaders useful in *S. cerevisiae*

include that from the mating factor  $\alpha$  polypeptide (MF $\alpha$ -1) and the hybrid leaders of EP-A-387 319. Such leaders (or signals) are cleaved by the yeast before the mature albumin is released into the surrounding medium. When the yeast strain lacks Kex2p activity (or equivalent) as well as being *yap3*, it may  
5 be advantageous to choose a secretion leader which need not be cleaved from the albumin by Kex2p. Such leaders include those of *S. cerevisiae* invertase (*SUC2*) disclosed in JP 62-096086 (granted as 91/036516), acid phosphatase (*PHO5*), the pre-sequence of MF $\alpha$ -1,  $\beta$ -glucanase (*BGL2*) and killer toxin; *S. diastaticus* glucoamylase II; *S. carlsbergensis*  $\alpha$ -galactosidase (*MEL1*); *K. lactis*  
10 killer toxin; and *Candida* glucoamylase.

Various non-limiting embodiments of the invention will now be described by way of example and with reference to the accompanying drawings in which:

15 Figure 1 is a general scheme for the construction of mutated rHA expression plasmids, in which HA is a human albumin coding sequence, L is a sequence encoding a secretion leader, P is the PRB1 promoter, T is the ADH1 terminator, amp is an ampicillin resistance gene and LEU2 is the leucine selectable marker;

20

Figure 2 is a drawing representing a Western blot analysis of mutant rHA secreted by *S. cerevisiae*, in which Track A represents the culture supernatant from DB1 *cir*<sup>o</sup> pAYE316 (normal rHA), Track B represents the culture supernatant from DB1 *cir*<sup>+</sup> pAYE464 (alteration 1), and Track C represents the  
25 culture supernatant from DB1 *cir*<sup>+</sup> pAYE468 (alteration 3);

Figure 3 is a scheme of the construction of pAYE515;

Figure 4 is a comparison of rHA fragment production by wild-type and  
30 protease-disrupted strains, presented as a drawing of an anti-HSA Western blot

of culture supernatant from shake flask cultures separated by non-reducing 10% SDS/PAGE, in which Track A corresponds to DB1 *cir*<sup>o</sup> pAYE316, Track B corresponds to DXY10 *cir*<sup>o</sup> pAYE316 (*yap3* strain), and Track C corresponds to ABB50 *cir*<sup>o</sup> pAYE316 (*yap3*, *kex2* strain);

5

Figure 5 is similar to Figure 4 but shows Coomassie Brilliant Blue stained 12.5% SDS Phastgel (Pharmacia) of culture supernatants from fed batch fermentations, namely Track D for the HSA standard, Track E for DB1 *cir*<sup>o</sup> pAYE316, Track F for DB1  $\Delta kex2$  *cir*<sup>o</sup> pAYE522, and Track G for DXY10 *cir*<sup>o</sup> pAYE522; and

10

Figure 6 is a scheme for the construction of pAYE519.

#### Detailed description of the invention

15

All standard recombinant DNA procedures are as described in Sambrook *et al* (1989) unless otherwise stated. The DNA sequences encoding HSA are derived from the cDNA disclosed in EP 201 239.

#### 20 **Example 1: Modification of the HSA cDNA.**

In order to investigate the role of endoproteases in the generation of rHA fragment, the HSA cDNA (SEQ1 (which includes a sequence encoding the artificial secretion leader sequence of WO 90/01063)) was modified by site-directed mutagenesis. Three separate changes were made to the HSA sequence (SEQ2). The first, using the mutagenic primer FOG1, changed the Arg<sup>410</sup> codon only, replacing it with an Ala codon, leaving intact the dibasic site, Lys<sup>413</sup>Lys<sup>414</sup>. The second change, using primer FOG2, changed the residues 407-409, including the C-terminal residues of fragment, from LeuLeuVal to AlaValAla. The third change, using the primer FOG3, altered residues 410-

30



414 from ArgTyrThrLysLys (SEQ3) to AlaTyrThrGlnGln (SEQ4). The oligonucleotides encoded not only the amino acid changes, but also conservative base changes that create either a *PvuII* or an *SpeI* restriction site in the mutants to facilitate detection of the changed sequences.

5

Single-stranded DNA of an M13mp19 clone, mp19.7 (EP 201 239; Figure 2), containing the HSA cDNA was used as the template for the mutagenesis reactions using the *In Vitro* Mutagenesis System, Version 2 (Amersham International plc) according to the manufacturer's instructions. Individual  
10 plaques were selected and sequenced to confirm the presence of the mutations. Double stranded RF DNA was then made from clones with the expected changes and the DNA bearing the mutation was excised on an *XbaI/SacI* fragment (Figure 1). This was used to replace the corresponding wild-type fragment of pAYE309 (EP 431 880; Figure 2). The presence of the mutated  
15 *XbaI/SacI* fragment within the plasmid was checked by digesting with *PvuII* or *SpeI* as appropriate. These *HindIII* fragments were excised and inserted into the expression vector pAYE219 (Figure 1) to generate the plasmids pAYE464 (alteration 1, R410A), pAYE470 (alteration 2, L407A, L408V, V409A) and pAYE468 (alteration 3, R410A, K413Q, K414Q). These expression plasmids  
20 comprise the *S. cerevisiae PRB1* promoter (WO 91/02057) driving expression of the HSA/MF $\alpha$ 1 leader sequence (WO 90/01063) fused in-frame with the mutated HA coding sequence which is followed by the *ADHI* transcription terminator. The plasmids also contain part of the 2 $\mu$ m plasmid to provide replication functions and the *LEU2* gene for selection of transformants.

25

pAYE464, pAYE470 and pAYE468 were introduced into *S. cerevisiae* DB1 *cir*<sup>+</sup> (*a*, *leu2*; Sleep *et al*, 1990) by transformation and individual transformants were grown for 3 days at 30°C in 10ml YEPS (1% w/v yeast extract, 2% w/v peptone, 2% w/v sucrose) and then the supernatants were examined by anti-HSA  
30 Western blot for the presence of the rHA fragment. The Western blots clearly

showed that fragment was still produced by the strains harbouring pAYE464, although the level was reduced slightly compared to the control expressing wild-type rHA. The mutations in the plasmid pAYE470 appeared to have no effect on the generation of fragment. However, DB1 cir<sup>+</sup> pAYE468 showed  
5 a novel pattern of HSA-related bands, with little or no fragment.

One example of each of DB1 cir<sup>+</sup> pAYE464 and DB1 cir<sup>+</sup> pAYE468 were grown to high cell density by fed batch culture in minimal medium in a fermenter (Collins, 1990). Briefly, a fermenter of 10L working volume was  
10 filled to 5L with an initial batch medium containing 50 mL/L of a concentrated salts mixture (Table 1), 10 mL/L of a trace elements solution (Table 2), 50 mL/L of a vitamins mixture (Table 3) and 20 g/L sucrose. An equal volume of feed medium containing 100 mL/L of the salts mixture, 20 mL/L of the trace elements mixture, 100 mL/L of vitamins solution and 500 g/L sucrose  
15 was held in a separate reservoir connected to the fermenter by a metering pump. The pH was maintained at  $5.7 \pm 0.2$  by the automatic addition of ammonium hydroxide or sulphuric acid, and the temperature was maintained at 30°C. The stirrer speed was adjusted to give a dissolved oxygen tension of >20% air saturation at 1 v/v/min air flow rate.

20

**Table 1. Salts Mixture**

Chemical	Concentration (g/L)
KH <sub>2</sub> PO <sub>4</sub>	114.0
MgSO <sub>4</sub>	12.0
CaCl <sub>2</sub> .6H <sub>2</sub> O	3.0
Na <sub>2</sub> EDTA	2.0

25

**Table 2. Trace Elements Solution**

	Chemical	Concentration (g/L)
5	ZnSO <sub>4</sub> ·7H <sub>2</sub> O	3.0
	FeSO <sub>4</sub> ·7H <sub>2</sub> O	10.0
	MnSO <sub>4</sub> ·4H <sub>2</sub> O	3.2
	CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.079
	H <sub>3</sub> BO <sub>3</sub>	1.5
10	KI	0.2
	Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.5
	CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.56
	H <sub>3</sub> PO <sub>4</sub>	75mL/L

**Table 3. Vitamins Solution**

	Chemical	Concentration (g/L)
15    20	Ca pantothenate	1.6
	Nicotinic acid	1.2
	m inositol	12.8
	Thiamine HCl	0.32
	Pyridoxine HCl	0.8
	Biotin	0.008

The fermenter was inoculated with 100 mL of an overnight culture of *S. cerevisiae* grown in buffered minimal medium (Yeast nitrogen base [without amino acids, without ammonium sulphate, Difco] 1.7 g/L, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 5 g/L, citric acid monohydrate 6.09 g/L, Na<sub>2</sub>HPO<sub>4</sub> 20.16 g/L, sucrose 20 g/L, pH6.5). The initial batch fermentation proceeded until the carbon source had been consumed, at which point the metering pump was switched on and the addition of feed was computer controlled (the micro MFCS system, B. Braun,

Melsungen, Germany) using an algorithm based on that developed by Wang *et al* (1979). A mass spectrometer was used in conjunction with the computer control system to monitor the off gases from the fermentation and to control the addition of feed to maintain a set growth rate (eg  $0.1 \text{ h}^{-1}$ ). Maximum  
5 conversion of carbon substrate into biomass is achieved by maintaining the respiratory coefficient below 1.2 (Collins, 1990) and, by this means, cell densities of approximately 100 g/L cell dry weight can be achieved. The culture supernatants were compared with those of a wild-type rHA producer by Coomassie-stained SDS/PAGE and by Western blot. These indicated (Figure  
10 2) that, whilst elimination of the monobasic Arg<sup>410</sup> (pAYE464) did reduce the level of the fragment by a useful amount, removal of both potential protease sites (pAYE468) almost abolished the 45kDa fragment.

The above data suggested that the generation of rHA fragment might be due to  
15 endoproteolytic attack, though the absence of an effect of removal of the potential Kex2p site Lys<sup>413</sup>Lys<sup>414</sup> (Sleep *et al*, 1990, and confirmed by other studies not noted here) unless combined with elimination of Arg<sup>410</sup>, had suggested a complex etiology. The reduction in the amount of fragment with the mutated rHA could in principle be due to an effect of the changes on the  
20 kinetics of folding of the molecule and not due to the removal of protease cleavage sites.

#### **Example 2: Disruption of the *YAP3* gene.**

25 The *YAP3* gene encoding yeast aspartyl protease 3 was mutated by the process of gene disruption (Rothstein 1983) which effectively deleted part of the *YAP3* coding sequence, thereby preventing the production of active Yap3p.

Four oligonucleotides suitable for PCR amplification of the 5' and 3' ends of  
30 the *YAP3* gene (Egel-Mitani *et al*, 1990) were synthesised using an Applied

Biosystems 380B Oligonucleotide Synthesiser. To assist the reader, we include as SEQ15 the sequence of the *YAP3* gene, of which 541-2250 is the coding sequence.

5 **5' end**

YAP3A: 5'-CGTCAGACCTTGCATGCAGCCAAGACACCCTCACATAGC-3' (SEQ5)

YAP3B: 5'-CCGTTACGTTCTGTGGTGGCATGCCCACTTCCAAGTCCACCG-3' (SEQ6)

10

**3' end**

YAP3C: 5'-GCGTCTCATAGTGGAAAAGCTTCTAAATACGACAACTTCCCC-3' (SEQ7)

15 YAP3D: 5'-CCCAAATGGTACCTGTGTCATCACTCGTTGGGATAATACC-3' (SEQ8)

PCR reactions were carried out to amplify individually the 5' and 3' ends of the *YAP3* gene from *S. cerevisiae* genomic DNA (Clontech Laboratories, Inc). Conditions were as follows: 2.5µg/ml genomic DNA, 5µg/ml of each primer, 20 denature at 94°C 61 seconds, anneal at 37°C 121 secs, extend at 72°C 181 secs for 40 cycles, followed by a 4°C soak, using a Perkin-Elmer-Cetus Thermal Cycler and a Perkin-Elmer-Cetus PCR kit according to the manufacturer's recommendations. Products were analysed by gel electrophoresis and were found to be of the expected size. The 5' fragment 25 was digested with *Sph*I and cloned into the *Sph*I site of pUC19HX (pUC19 lacking a *Hind*III site) to give pAYE511 (Figure 3), in which the orientation is such that *YAP3* would be transcribed towards the *Kpn*I site of the pUC19HX polylinker. The 3' *YAP3* fragment was digested with *Hind*III and *Asp*718 (an isoschizomer of *Kpn*I) and ligated into pUC19 digested with *Hind*III/*Asp*718 to 30 give pAYE512. Plasmid DNA sequencing was carried out on the inserts to confirm that the desired sequences had been cloned. The *Hind*III/*Asp*718 fragment of pAYE512 was then subcloned into the corresponding sites of pAYE511 to give pAYE513 (Fig 3), in which the 5' and 3' regions of *YAP3*



are correctly orientated with a unique *Hind*III site between them. The *URA3* gene was isolated from YEp24 (Botstein *et al*, 1979) as a *Hind*III fragment and then inserted into this site to give pAYE515 (Fig 3), with *URA3* flanked by the 5' and 3' regions of *YAP3*, and transcribed in the opposite direction to *YAP3*.

5

A *ura3* derivative of strain DB1 *cir*<sup>o</sup> pAYE316 (Sleep *et al*, 1991) was obtained by random chemical mutagenesis and selection for resistance to 5-fluoro-orotic acid (Boeke *et al*, 1987). The strain was grown overnight in 100 mL buffered minimal medium and the cells were collected by centrifugation and then washed once with sterile water. The cells were then resuspended in 10 mL sterile water and 2 mL aliquots were placed in separate 15 mL Falcon tubes. A 5 mg/mL solution of N-methyl-N'-nitro-N-nitrosoguanidine (NTG) was then added to the tubes as follows: 0  $\mu$ L, 20  $\mu$ L, 40  $\mu$ L, 80  $\mu$ L or 160  $\mu$ L. The cells were then incubated at 30°C for 30 min and then centrifuged and washed three times with sterile water. Finally, the cells were resuspended in 1 mL YEP (1% w/v yeast extract, 2% w/v Bacto peptone) and stored at 4°C. The percentage of cells that survived the mutagenic treatment was determined by spreading dilutions of the samples on YEP plates containing 2% w/v sucrose and incubating at 30°C for 3 days. Cells from the treatment which gave approximately 50% survival were grown on YEP plates containing 2% w/v sucrose and then replica-plated onto YNB minimal medium containing 2% w/v sucrose and supplemented with 5-fluoro-orotic acid (1 mg/mL) and uracil (50  $\mu$ g/mL). Colonies able to grow on this medium were purified, tested to verify that they were unable to grow in the absence of uracil supplementation and that this defect could be corrected by introduction of the *URA3* gene by transformation. One such strain, DBU3 *cir*<sup>o</sup> pAYE316, was transformed with the *Sph*I/*Asp*718 *YAP3-URA3-YAP3* fragment of pAYE515 with selection for Ura<sup>+</sup> colonies. A Southern blot of digested genomic DNA of a number of transformants was probed with the 5' and 3' ends of the *YAP3* gene and confirmed the disruption of the *YAP3* gene. An anti-HSA Western blot of

30



YEPS shake-flask supernatants of two transformants indicated that disruption of *YAP3* markedly reduced rHA fragment levels.

One *yap3* derivative of DBU3 cir<sup>o</sup> pAYE316, designated DXY10 cir<sup>o</sup> pAYE316, was grown several times by fed-batch fermentation in minimal medium to high cell dry weight. When supernatants were examined by Coomassie-stained PAGE and anti-HSA Western blot (Figs 4 and 5), the reduction in the level of rHA 45kDa fragment was clearly apparent; estimates of the amount of the degradation product vary from  $\frac{1}{3}$  to  $\frac{1}{5}$  of the levels seen with the *YAP3* parent. The amount of rHA produced was not adversely affected by the *yap3* mutation, indeed DXY10 cir<sup>o</sup> pAYE316 was found to produce 30-50% more rHA than the *YAP3* equivalent, DB1 cir<sup>o</sup> pAYE316. Despite the fact that cleavage of the leader sequence from the HA sequence is C-terminal to a pair of basic residues, the rHA was found to have the correct N-terminus.

The fermentation broth was centrifuged to remove the cells and then subject to affinity chromatographic purification as follows. The culture supernatant was passed through a Cibacron Blue F3GA Sepharose column (Pharmacia) which was then washed with 0.1M phosphate glycine buffer, pH8.0. The rHA was then eluted from the column with 2M NaCl, 0.1M phosphate glycine, pH8.0, at which point it was >95% pure. It may be purified further by techniques known in the art.

The albumin may alternatively be purified from the culture medium by any of the variety of known techniques for purifying albumin from serum or fermentation culture medium, for example those disclosed in WO 92/04367, Maurel *et al* (1989), Curling (1980) and EP 524 681.

**Example 3: Disruption of the *KEX2* gene in a *yap3* strain.**

To construct a strain lacking both Yap3p and Kex2p activity, a *lys2* derivative of yeast strain DXY10 *cir*<sup>o</sup> (pAYE316) was obtained by random chemical  
5 mutagenesis and selection for resistance to  $\alpha$ -amino adipate (Barnes and Thorner, 1985). Cells were mutagenised as in Example 2 and then plated on YNB minimal medium containing 2% w/v sucrose and supplemented with 2 mg/mL DL- $\alpha$ -amino adipate as the sole nitrogen source and 30  $\mu$ g/mL lysine. Colonies able to grow on this medium were purified and tested to verify that  
10 they were unable to grow in the absence of lysine supplementation and that this defect could be corrected by the introduction of the *LYS2* gene by transformation. This strain was then mutated by the process of gene disruption which effectively disrupted part of the *KEX2* coding sequence, thereby preventing production of active Kex2p. To assist the reader, the sequence of  
15 the *KEX2* gene is reproduced herein as SEQ14, of which 1329-3773 is the coding sequence.

Four oligonucleotides suitable for PCR amplification of the 5' and 3' ends of the *KEX2* gene (Fuller *et al*, 1989) were synthesised using an Applied  
20 Biosystems 380B Oligonucleotide Synthesiser.

**5' end**

KEX2A: 5'-CCATCTGGATCCAATGGTGCTTTGGCCAAATAAATAGTTTCAGC-3'  
(SEQ9)  
25 KEX2B: 5'-GCTTCTTTTACCGGTAACAAGCTTGAGTCCATTGG-3'  
(SEQ10)

**3' end**

KEX2C: 5'-GGTAAGGTTTAGTCGACCTATTTTTTGTGTTTGTCTGC-3'  
(SEQ11)  
30 KEX2D: 5'-GGAAACGTATGAATTCGATATCATTGATACAGACTCTGAGTACG-3'  
(SEQ12)

PCR reactions were carried out to amplify individually the 5' and 3' ends of the *KEX2* gene from *S. cerevisiae* genomic DNA (Clontech Laboratories Inc). Conditions were as follows: 2.5 µg/ml genomic DNA, 5 µg/ml of each primer, denature 94°C 61s, anneal 37°C 121s, extend 72°C 181s for 40 cycles, followed by a 4°C soak, using a Perkin-Elmer-Cetus Thermal Cycler and a Perkin-Elmer-Cetus PCR kit according to the manufacturer's recommendations. Products were analysed by gel electrophoresis and were found to be of the expected size (0.9 kb for the 5' product and 0.62 kb for the 3' product). The 5' product was digested with *Bam*HI and *Hind*III and the 3' product was digested with *Hind*III and *Sal*I and then the two fragments were together cloned into pUC19HX digested with *Bam*HI and *Sal*I. A 4.8 kb *Hind*III fragment comprising the *S. cerevisiae* *LYS2* gene (Barnes & Thorner, 1985) was then inserted into the resulting plasmid at *Hind*III (ie between the two *KEX2* fragments) to form pAYE519 (Fig 6).

The *lys2* derivative of DXY10 cir<sup>o</sup> (pAYE316), *lys2-16*, was transformed with the 6.0 kb *KEX2-LYS2-KEX2* fragment of pAYE519, selecting for Lys<sup>+</sup> colonies. A Southern blot of digested genomic DNA of a number of transformants was probed with the 5' and 3' ends of the *KEX2* gene and confirmed the disruption of the *KEX2* gene. An anti-HSA Western blot of YEPS shake-flask culture supernatants of these transformants indicated that disruption of *KEX2* in a *yap3* strain reduced the level of rHA fragment still further, despite the lack of an effect of disruption of *KEX2* alone in Example 4 below. Analysis of the rHA produced by one such strain, ABB50, indicated that the leader sequence was incorrectly processed, leading to an abnormal N-terminus.

The strain ABB50 (pAYE316) was cured of its plasmid (Sleep *et al*, 1991) and transformed with a similar plasmid, pAYE522, in which the hybrid leader sequence was replaced by the *S. cerevisiae* invertase (*SUC2*) leader sequence

such that the encoded leader and the junction with the HSA sequence were as follows:

	MLLQAFLFLLAGFAAKISA↓DAHKS	(SEQ13)
5	Invertase leader	HSA

In this construct, cleavage of the leader sequence from HSA does not rely upon activity of the Kex2 protease. The strain ABB50 (pAYE522) was found to produce rHA with a similarly very low level of rHA fragment, but in this instance the N-terminus corresponded to that of serum-derived HSA, ie there was efficient and precise removal of the leader sequence.

**Example 4: Disruption of the *KEX2* gene alone (Comparative Example).**

By a similar method to that disclosed in Example 3 the *KEX2* gene was disrupted in *S. cerevisiae*. This strain had the Yap3p proteolytic activity and was therefore not within the scope of the invention. When this strain was grown in fed batch fermentation the rHA produced contained similar amounts of fragment to that produced by strains with an intact *KEX2* gene. In addition, the overall level of rHA was reduced and the leader sequence was not correctly processed, leading to an abnormal N-terminus.

**Example 5: Identification of equivalent protease in *Pichia*.**

As noted above, non-*Saccharomyces* yeast similarly produce the undesirable fragment of rHA and therefore have the Yap3p proteolytic activity. We have confirmed this by performing Southern hybridisations of *Pichia angusta* DNA, using the *S. cerevisiae* *YAP3* gene as a probe. A specific DNA fragment was identified, showing that, not only is the Yap3p proteolytic activity present in *P. angusta*, but a specific homologue of the *YAP3* gene is present also.

The method of Southern hybridization used for detection of the *YAP3* homologue can be adapted to clone the gene sequence from a genomic DNA library of *Pichia* DNA using standard procedures (Sambrook *et al.*, 1989). Disruption of the *YAP3* homologue in *Pichia sp.* can be achieved using similar  
5 techniques to those used above for *Saccharomyces* (Cregg and Madden, 1987).

**References** (all incorporated by reference)

- Azaryan, A.V. *et al* (1993) *J. Biol. Chem.* **268**, 11968-11975.
- Barnes, D.A. and Thorner, J. (1985) *In* Gene Manipulations in Fungi (Bennett, J.W. and Lasure, L.L., eds), pp. 197-226, Academic Press.
- 5 Boeke, J.D. *et al* (1987) *Methods Enzymol.* **154**, 164-175.
- Botstein, D. *et al* (1979) *Gene* **8**, 17-24.
- Botstein & Shortle (1985) *Science* **229**, 193-210.
- Bourbonnais, Y. *et al* (1991) *J. Biol. Chem.* **266**, 13203-13209.
- 10 Bourbonnais, Y. *et al* (1993) *EMBO J.* **12**, 285-294.
- Cawley, N.X. *et al* (1993) *FEBS Lett.* **332**, 273-276.
- Clerc *et al* (1994) *J. Chromat. B.* **662**, 245-259.
- Collins, S.H. (1990) *In* Protein Production by Biotechnology (Harris, T.J.R., ed) pp. 61-77, Elsevier Science Publishers, Barking, Essex.
- 15 Cregg, J.M. and Madden, K.R. (1987) *In* Biological Research on Industrial Yeasts, Vol. II, Stewart, G.G., Russell, I., Klein, R.D. and Hiebsch, R.R. (Eds) CRC Press, Boca Raton, FL.
- Cregg *et al* (1993) *Bio/Technology* **11**, 905-910.
- Curling (1980) "Albumin Purification by Ion Exchange Chromatography", in
- 20 "Methods of Plasma Protein Purification", Ed. Curling, J.M., Academic Press, London.
- Enderlin, C.S. & Ogrydziak, D.M. (1994) *Yeast* **10**, 67-79.
- Fleer, R. *et al* (1991) *Bio/Technology* **9**, 968-975.
- Fuller, R.S. *et al* (1989) *Proc. Natl. Acad. Sci. USA* **86**, 1434-1438.
- 25 Geisow, M.J. *et al* (1991) *In* Techniques in Protein Chemistry II, pp. 567-572, Academic Press, Inc.
- Gellissen *et al* (1992) *Tibtech* **10**, 413-417.
- Hoffmann & Winston (1990) *Genetics* **124**, 807-816.
- Maundrell (1990) *J. Biol. Chem.* **265**, 10857-10864.
- 30 Maurel *et al* (1989) "Biotechnology of Plasma Proteins", *Colloque INSERM*



175, 19-24.

Romanos, M.A. (1992) *Yeast* **8**, 423-488.

Rothstein, R.J. (1983) *Methods Enzymol.* **101**, 203-211.

5 Sambrook, J. *et al* (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sleep, D. *et al* (1990) *Bio/Technology* **8**, 42-46.

Sleep, D. *et al* (1991) *Bio/Technology* **9**, 183-187.

Tanguy-Rougeau, C. *et al* (1988) *FEBS Lett.* **234**, 464-470

Wang, H.Y. *et al* (1979) *Biotech. & Bioeng.* **21**, 975.

10 Winston, F. *et al* (1983) *Methods Enzymol.* **101**, 211-228.

## 26

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

- (A) NAME: Delta Biotechnology Limited
- (B) STREET: Castle Court, Castle Boulevard
- (C) CITY: Nottingham
- (D) STATE: Nottinghamshire
- (E) COUNTRY: United Kingdom
- (F) POSTAL CODE (ZIP): NG7 1FD

(ii) TITLE OF INVENTION: Yeast strains and modified albumins

(iii) NUMBER OF SEQUENCES: 15

## (iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1830 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 73..1827

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATGAAGTGGG TAAGCTTTAT TTCCCTTCTT TTTCTCTTTA GCTCGGCTTA TTCCAGGAGC	60
TTGGATAAAA GA GAT GCA CAC AAG AGT GAG GTT GCT CAT CGG TTT AAA	108
Asp Ala His Lys Ser Glu Val Ala His Arg Phe Lys	
1 5 10	
GAT TTG GGA GAA GAA AAT TTC AAA GCC TTG GTG TTG ATT GCC TTT GCT	156
Asp Leu Gly Glu Glu Asn Phe Lys Ala Leu Val Leu Ile Ala Phe Ala	
15 20 25	
CAG TAT CTT CAG CAG TGT CCA TTT GAA GAT CAT GTA AAA TTA GTG AAT	204
Gln Tyr Leu Gln Gln Cys Pro Phe Glu Asp His Val Lys Leu Val Asn	
30 35 40	
GAA GTA ACT GAA TTT GCA AAA ACA TGT GTT GCT GAT GAG TCA GCT GAA	252
Glu Val Thr Glu Phe Ala Lys Thr Cys Val Ala Asp Glu Ser Ala Glu	
45 50 55 60	

27

AAT	TGT	GAC	AAA	TCA	CTT	CAT	ACC	CTT	TTT	GGA	GAC	AAA	TTA	TGC	ACA	300
Asn	Cys	Asp	Lys	Ser	Leu	His	Thr	Leu	Phe	Gly	Asp	Lys	Leu	Cys	Thr	
				65					70					75		
GTT	GCA	ACT	CTT	CGT	GAA	ACC	TAT	GGT	GAA	ATG	GCT	GAC	TGC	TGT	GCA	348
Val	Ala	Thr	Leu	Arg	Glu	Thr	Tyr	Gly	Glu	Met	Ala	Asp	Cys	Cys	Ala	
			80					85					90			
AAA	CAA	GAA	CCT	GAG	AGA	AAT	GAA	TGC	TTC	TTG	CAA	CAC	AAA	GAT	GAC	396
Lys	Gln	Glu	Pro	Glu	Arg	Asn	Glu	Cys	Phe	Leu	Gln	His	Lys	Asp	Asp	
		95					100					105				
AAC	CCA	AAC	CTC	CCC	CGA	TTG	GTG	AGA	CCA	GAG	GTT	GAT	GTG	ATG	TGC	444
Asn	Pro	Asn	Leu	Pro	Arg	Leu	Val	Arg	Pro	Glu	Val	Asp	Val	Met	Cys	
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ACT	GCT	TTT	CAT	GAC	AAT	GAA	GAG	ACA	TTT	TTG	AAA	AAA	TAC	TTA	TAT	492
Thr	Ala	Phe	His	Asp	Asn	Glu	Glu	Thr	Phe	Leu	Lys	Lys	Tyr	Leu	Tyr	
125					130					135					140	
GAA	ATT	GCC	AGA	AGA	CAT	CCT	TAC	TTT	TAT	GCC	CCG	GAA	CTC	CTT	TTC	540
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TTT	GCT	AAA	AGG	TAT	AAA	GCT	GCT	TTT	ACA	GAA	TGT	TGC	CAA	GCT	GCT	588
Phe	Ala	Lys	Arg	Tyr	Lys	Ala	Ala	Phe	Thr	Glu	Cys	Cys	Gln	Ala	Ala	
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GAT	AAA	GCT	GCC	TGC	CTG	TTG	CCA	AAG	CTC	GAT	GAA	CTT	CGG	GAT	GAA	636
Asp	Lys	Ala	Ala	Cys	Leu	Leu	Pro	Lys	Leu	Asp	Glu	Leu	Arg	Asp	Glu	
		175					180					185				
GGG	AAG	GCT	TCG	TCT	GCC	AAA	CAG	AGA	CTC	AAG	TGT	GCC	AGT	CTC	CAA	684
Gly	Lys	Ala	Ser	Ser	Ala	Lys	Gln	Arg	Leu	Lys	Cys	Ala	Ser	Leu	Gln	
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AAA	TTT	GGA	GAA	AGA	GCT	TTC	AAA	GCA	TGG	GCA	GTA	GCT	CGC	CTG	AGC	732
Lys	Phe	Gly	Glu	Arg	Ala	Phe	Lys	Ala	Trp	Ala	Val	Ala	Arg	Leu	Ser	
205					210				215						220	
CAG	AGA	TTT	CCC	AAA	GCT	GAG	TTT	GCA	GAA	GTT	TCC	AAG	TTA	GTG	ACA	780
Gln	Arg	Phe	Pro	Lys	Ala	Glu	Phe	Ala	Glu	Val	Ser	Lys	Leu	Val	Thr	
			225					230						235		
GAT	CTT	ACC	AAA	GTC	CAC	ACG	GAA	TGC	TGC	CAT	GGA	GAT	CTG	CTT	GAA	828
Asp	Leu	Thr	Lys	Val	His	Thr	Glu	Cys	Cys	His	Gly	Asp	Leu	Leu	Glu	
			240				245						250			
TGT	GCT	GAT	GAC	AGG	GCG	GAC	CTT	GCC	AAG	TAT	ATC	TGT	GAA	AAT	CAA	876
Cys	Ala	Asp	Asp	Arg	Ala	Asp	Leu	Ala	Lys	Tyr	Ile	Cys	Glu	Asn	Gln	
		255					260					265				
GAT	TCG	ATC	TCC	AGT	AAA	CTG	AAG	GAA	TGC	TGT	GAA	AAA	CCT	CTG	TTG	924
Asp	Ser	Ile	Ser	Ser	Lys	Leu	Lys	Glu	Cys	Cys	Glu	Lys	Pro	Leu	Leu	
	270					275					280					
GAA	AAA	TCC	CAC	TGC	ATT	GCC	GAA	GTG	GAA	AAT	GAT	GAG	ATG	CCT	GCT	972
Glu	Lys	Ser	His	Cys	Ile	Ala	Glu	Val	Glu	Asn	Asp	Glu	Met	Pro	Ala	
285					290					295					300	
GAC	TTG	CCT	TCA	TTA	GCT	GCT	GAT	TTT	GTT	GAA	AGT	AAG	GAT	GTT	TGC	1020
Asp	Leu	Pro	Ser	Leu	Ala	Ala	Asp	Phe	Val	Glu	Ser	Lys	Asp	Val	Cys	
			305						310					315		
AAA	AAC	TAT	GCT	GAG	GCA	AAG	GAT	GTC	TTC	CTG	GGC	ATG	TTT	TTG	TAT	1068
Lys	Asn	Tyr	Ala	Glu	Ala	Lys	Asp	Val	Phe	Leu	Gly	Met	Phe	Leu	Tyr	

GAA Glu	TAT Tyr	GCA Ala 335	AGA Arg	AGG Arg	CAT His	CCT Pro	GAT Asp 340	TAC Tyr	TCT Ser	GTC Val	GTG Val	CTG Leu 345	CTG Leu	CTG Leu	AGA Arg	1116
CTT Leu	GCC Ala 350	AAG Lys	ACA Thr	TAT Tyr	GAA Glu	ACC Thr 355	ACT Thr	CTA Leu	GAG Glu	AAG Lys	TGC Cys 360	TGT Cys	GCC Ala	GCT Ala	GCA Ala	1164
GAT Asp 365	CCT Pro	CAT His	GAA Glu	TGC Cys	TAT Tyr 370	GCC Ala	AAA Lys	GTG Val	TTC Phe	GAT Asp 375	GAA Glu	TTT Phe	AAA Lys	CCT Pro	CTT Leu 380	1212
GTG Val	GAA Glu	GAG Glu	CCT Pro	CAG Gln 385	AAT Asn	TTA Leu	ATC Ile	AAA Lys	CAA Gln 390	AAT Asn	TGT Cys	GAG Glu	CTT Leu	TTT Phe 395	GAG Glu	1260
CAG Gln	CTT Leu	GGA Gly	GAG Glu 400	TAC Tyr	AAA Lys	TTC Phe	CAG Gln	AAT Asn 405	GCG Ala	CTA Leu	TTA Leu	GTT Val	CGT Arg 410	TAC Tyr	ACC Thr	1308
AAG Lys	AAA Lys	GTA Val 415	CCC Pro	CAA Gln	GTG Val	TCA Ser	ACT Thr 420	CCA Pro	ACT Thr	CTT Leu	GTA Val	GAG Glu 425	GTC Val	TCA Ser	AGA Arg	1356
AAC Asn	CTA Leu 430	GGA Gly	AAA Lys	GTG Val	GGC Gly	AGC Ser 435	AAA Lys	TGT Cys	TGT Cys	AAA Lys	CAT His 440	CCT Pro	GAA Glu	GCA Ala	AAA Lys	1404
AGA Arg 445	ATG Met	CCC Pro	TGT Cys	GCA Ala	GAA Glu 450	GAC Asp	TAT Tyr	CTA Leu	TCC Ser	GTG Val 455	GTC Val	CTG Leu	AAC Asn	CAG Gln	TTA Leu 460	1452
TGT Cys	GTG Val	TTG Leu	CAT His	GAG Glu 465	AAA Lys	ACG Thr	CCA Pro	GTA Val	AGT Ser 470	GAC Asp	AGA Arg	GTC Val	ACC Thr	AAA Lys 475	TGC Cys	1500
TGC Cys	ACA Thr	GAA Glu	TCC Ser 480	TTG Leu	GTG Val	AAC Asn	AGG Arg 485	CGA Arg	CCA Pro	TGC Cys	TTT Phe	TCA Ser	GCT Ala 490	CTG Leu	GAA Glu	1548
GTC Val	GAT Asp	GAA Glu 495	ACA Thr	TAC Tyr	GTT Val	CCC Pro	AAA Lys 500	GAG Glu	TTT Phe	AAT Asn	GCT Ala 505	GAA Glu	ACA Thr	TTC Phe	ACC Thr	1596
TTC Phe	CAT His 510	GCA Ala	GAT Asp	ATA Ile	TGC Cys	ACA Thr 515	CTT Leu	TCT Ser	GAG Glu	AAG Lys	GAG Glu 520	AGA Arg	CAA Gln	ATC Ile	AAG Lys	1644
AAA Lys 525	CAA Gln	ACT Thr	GCA Ala	CTT Leu	GTT Val 530	GAG Glu	CTC Leu	GTG Val	AAA Lys	CAC His 535	AAG Lys	CCC Pro	AAG Lys	GCA Ala	ACA Thr 540	1692
AAA Lys	GAG Glu	CAA Gln	CTG Leu	AAA Lys 545	GCT Ala	GTT Val	ATG Met	GAT Asp	GAT Asp 550	TTC Phe	GCA Ala	GCT Ala	TTT Phe	GTA Val 555	GAG Glu	1740
AAG Lys	TGC Cys	TGC Cys	AAG Lys 560	GCT Ala	GAC Asp	GAT Asp	AAG Lys	GAG Glu 565	ACC Thr	TGC Cys	TTT Phe	GCC Ala	GAG Glu 570	GAG Glu	GGT Gly	1788
AAA Lys	AAA Lys	CTT Leu 575	GTT Val	GCT Ala	GCA Ala	AGT Ser	CAA Gln 580	GCT Ala	GCC Ala	TTA Leu	GGC Gly	TTA Leu 585	TAA			1830

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 585 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```

Asp Ala His Lys Ser Glu Val Ala His Arg Phe Lys Asp Leu Gly Glu
 1          5          10          15
Glu Asn Phe Lys Ala Leu Val Leu Ile Ala Phe Ala Gln Tyr Leu Gln
          20          25          30
Gln Cys Pro Phe Glu Asp His Val Lys Leu Val Asn Glu Val Thr Glu
          35          40          45
Phe Ala Lys Thr Cys Val Ala Asp Glu Ser Ala Glu Asn Cys Asp Lys
          50          55          60
Ser Leu His Thr Leu Phe Gly Asp Lys Leu Cys Thr Val Ala Thr Leu
 65          70          75          80
Arg Glu Thr Tyr Gly Glu Met Ala Asp Cys Cys Ala Lys Gln Glu Pro
          85          90          95
Glu Arg Asn Glu Cys Phe Leu Gln His Lys Asp Asp Asn Pro Asn Leu
          100          105          110
Pro Arg Leu Val Arg Pro Glu Val Asp Val Met Cys Thr Ala Phe His
          115          120          125
Asp Asn Glu Glu Thr Phe Leu Lys Lys Tyr Leu Tyr Glu Ile Ala Arg
          130          135          140
Arg His Pro Tyr Phe Tyr Ala Pro Glu Leu Leu Phe Phe Ala Lys Arg
          145          150          155          160
Tyr Lys Ala Ala Phe Thr Glu Cys Cys Gln Ala Ala Asp Lys Ala Ala
          165          170          175
Cys Leu Leu Pro Lys Leu Asp Glu Leu Arg Asp Glu Gly Lys Ala Ser
          180          185          190
Ser Ala Lys Gln Arg Leu Lys Cys Ala Ser Leu Gln Lys Phe Gly Glu
          195          200          205
Arg Ala Phe Lys Ala Trp Ala Val Ala Arg Leu Ser Gln Arg Phe Pro
          210          215          220
Lys Ala Glu Phe Ala Glu Val Ser Lys Leu Val Thr Asp Leu Thr Lys
          225          230          235          240
Val His Thr Glu Cys Cys His Gly Asp Leu Leu Glu Cys Ala Asp Asp
          245          250          255
Arg Ala Asp Leu Ala Lys Tyr Ile Cys Glu Asn Gln Asp Ser Ile Ser
          260          265          270
Ser Lys Leu Lys Glu Cys Cys Glu Lys Pro Leu Leu Glu Lys Ser His
          275          280          285
Cys Ile Ala Glu Val Glu Asn Asp Glu Met Pro Ala Asp Leu Pro Ser

```

30

290					295					300					
Leu 305	Ala	Ala	Asp	Phe	Val 310	Glu	Ser	Lys	Asp	Val 315	Cys	Lys	Asn	Tyr	Ala 320
Glu	Ala	Lys	Asp	Val 325	Phe	Leu	Gly	Met	Phe 330	Leu	Tyr	Glu	Tyr	Ala 335	Arg
Arg	His	Pro	Asp 340	Tyr	Ser	Val	Val	Leu 345	Leu	Leu	Arg	Leu	Ala 350	Lys	Thr
Tyr	Glu	Thr 355	Thr	Leu	Glu	Lys	Cys 360	Cys	Ala	Ala	Ala	Asp 365	Pro	His	Glu
Cys	Tyr 370	Ala	Lys	Val	Phe	Asp 375	Glu	Phe	Lys	Pro	Leu 380	Val	Glu	Glu	Pro
Gln 385	Asn	Leu	Ile	Lys	Gln 390	Asn	Cys	Glu	Leu	Phe 395	Glu	Gln	Leu	Gly	Glu 400
Tyr	Lys	Phe	Gln	Asn 405	Ala	Leu	Leu	Val	Arg 410	Tyr	Thr	Lys	Lys	Val 415	Pro
Gln	Val	Ser	Thr 420	Pro	Thr	Leu	Val	Glu 425	Val	Ser	Arg	Asn	Leu 430	Gly	Lys
Val	Gly	Ser 435	Lys	Cys	Cys	Lys	His 440	Pro	Glu	Ala	Lys	Arg 445	Met	Pro	Cys
Ala	Glu	Asp	Tyr	Leu	Ser	Val 455	Val	Leu	Asn	Gln	Leu 460	Cys	Val	Leu	His
Glu 465	Lys	Thr	Pro	Val	Ser 470	Asp	Arg	Val	Thr	Lys 475	Cys	Cys	Thr	Glu	Ser 480
Leu	Val	Asn	Arg	Arg 485	Pro	Cys	Phe	Ser	Ala 490	Leu	Glu	Val	Asp	Glu	Thr 495
Tyr	Val	Pro	Lys 500	Glu	Phe	Asn	Ala	Glu 505	Thr	Phe	Thr	Phe	His 510	Ala	Asp
Ile	Cys	Thr 515	Leu	Ser	Glu	Lys	Glu 520	Arg	Gln	Ile	Lys	Lys 525	Gln	Thr	Ala
Leu 530	Val	Glu	Leu	Val	Lys	His 535	Lys	Pro	Lys	Ala	Thr 540	Lys	Glu	Gln	Leu
Lys 545	Ala	Val	Met	Asp	Asp 550	Phe	Ala	Ala	Phe	Val 555	Glu	Lys	Cys	Cys	Lys 560
Ala	Asp	Asp	Lys	Glu 565	Thr	Cys	Phe	Ala	Glu 570	Glu	Gly	Lys	Lys	Leu 575	Val
Ala	Ala	Ser	Gln	Ala	Ala	Leu	Gly	Leu 585							

## (2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 5 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide



31

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Arg	Tyr	Thr	Lys	Lys
1				5

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 5 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Ala	Tyr	Thr	Gln	Gln
1				5

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 39 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CGTCAGACCT TGCATGCAGC CAAGACACCC TCACATAGC

39

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 42 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CCGTTACGTT CTGTGGTGGC ATGCCCACTT CCAAGTCCAC CG

42

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 42 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GCGTCTCATA GTGGAAAAGC TTCTAAATAC GACAACTTCC CC

42

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 41 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CCCAAATGG TACCTGTGTC ATCACTCGTT GGGATAATAC C

41

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 44 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CCATCTGGAT CCAATGGTGC TTGGCCAAA TAAATAGTTT CAGC

44

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GCTTCTTTTA CCGTAACAA GCTTGAGTCC ATTGG

35

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 37 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GGTAAGGTTT AGTCGACCTA TTTTGTGTTT TGTCTGC

37

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GGAAACGTAT GAATTCGATA TCATTGATAC AGACTCTGAG TACG

44

(2) INFORMATION FOR SEQ ID NO: 13:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 24 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Met	Leu	Leu	Gln	Ala	Phe	Leu	Phe	Leu	Leu	Ala	Gly	Phe	Ala	Ala	Lys
1			5					10						15	

Ile	Ser	Ala	Asp	Ala	His	Lys	Ser
			20				

(2) INFORMATION FOR SEQ ID NO: 14:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 4106 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Saccharomyces cerevisiae*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GAATTCTCTG TTGACTACTA AACTGAGAGA ATTTGCCGAG ACTCTAAGAA CAGCTTTGAA	60
AGAGCGTTCT GCCGATGATT CCATAATTGT CACTCTGAGA GAGCAAATGC AAAGAGAAAT	120
CTTCAGGTTG ATGTCGTTGT TCATGGACAT ACCTCCAGTG CAACCAAACG AGCAATTCAC	180
TTGGGAATAC GTTGACAAAG ACAAGAAAAT CCACACTATC AAATCGACTC CGTTAGAATT	240
TGCCTCCAAA TACGCAAAAT TGGACCCTTC CACGCCAGTC TCATTGATCA ATGATCCAAG	300
ACACCATATG GTAAATTAAT TAAGATCGAT CGTTTAGGAA ACGTCCTTGG CGGAGATGCC	360
GTGATTTACT TAAATGTTGA CAATGAAACA CTATCTAAAT TGGTTGTTAA GAGATTACAA	420
AATAACAAAG CTGTCTTTTT TGGATCTCAC ACTCCAAAGT TCATGGACAA GAAAACTGGT	480
GTCATGGATA TTGAATTGTG GAACTATCCT GCCATGGCTA TAATTTACCT CAGCAAAAGG	540
CATCCGGTAT TAGATACCAT GAAAGTTTGA TGAATCATGC TATGTTGGAT CACTGGCTGC	600
CACGTCGATG AAACGTCTAA ATTACCACTT CGCTACCGTC TGAAAATTCC TGGGGTAAAG	660

ACTCCGGTAA	AGACGGATTA	TACGTGATGA	CTCAAAAGTA	CTTCGAGGAG	TACTGCTTTC	720
AAATTGTGGT	CGATATCAAT	GAATTGCCAA	AAGAGCTGGC	TTCAAAATTC	ACCTCAGGTA	780
AGGAAGAGCC	GATTGTCTTG	CCCATCTGGA	CCCAATGGTG	CTTTGGCCAA	ATAAATAGTT	840
TCAGCAGCTC	TGATGTAGAT	ACACGTATCT	CGACATGTTT	TATTTTACT	ATACATACAT	900
AAAAGAAATA	AAAAATGATA	ACGTGTATAT	TATTATTCAT	ATAATCAATG	AGGGTCATTT	960
TCTGAAACGC	AAAAACGGT	AAATGGAAAA	AAAATAAAGA	TAGAAAAAGA	AAACAAACAA	1020
AGGAAAGGTT	AGCATATTAA	ATAACTGAGC	TGATACTTCA	ACAGCATCGC	TGAAGAGAAC	1080
AGTATTGAAA	CCGAAACATT	TTCTAAAGGC	AAACAAGGTA	CTCCATATTT	GCTGGACGTG	1140
TTCTTTCTCT	CGTTTCATAT	GCATAATTCT	GTCATAAGCC	TGTTCTTTTT	CCTGGCTTAA	1200
ACATCCCGTT	TTGTAAAAGA	GAAATCTATT	CCACATATTT	CATTCATTCG	GCTACCATAC	1260
TAAGGATAAA	CTAATCCCGT	TGTTTTTTGG	CCTCGTCACA	TAATTATAAA	CTACTAACCC	1320
ATTATCAGAT	GAAAGTGAGG	AAATATATTA	CTTTATGCTT	TTGGTGGGCC	TTTTCAACAT	1380
CCGCTCTTGT	ATCATCACAA	CAAATTCCAT	TGAAGGACCA	TACGTCACGA	CAGTATTTTG	1440
CTGTAGAAAG	CAATGAAACA	TTATCCCGCT	TGGAGGAAAT	GCATCCAAAT	TGGAAATATG	1500
AACATGATGT	TCGAGGGCTA	CCAAACCATT	ATGTTTTTTC	AAAAGAGTTG	CTAAAATTGG	1560
GCAAAAGATC	ATCATTAGAA	GAGTTACAGG	GGGATAACAA	CGACCACATA	TTATCTGTCC	1620
ATGATTTATT	CCCGCGTAAC	GACCTATTTA	AGAGACTACC	GGTGCCTGCT	CCACCAATGG	1680
ACTCAAGCTT	GTTACCGGTA	AAAGAAGCTG	AGGATAAACT	CAGCATAAAT	GATCCGCTTT	1740
TTGAGAGGCA	GTGGCACTTG	GTCAATCCAA	GTTTTCTG	CAGTGATATA	AATGTTCTTG	1800
ATCTGTGGTA	CAATAATATT	ACAGGCGCAG	GGGTCGTGGC	TGCCATTGTT	GATGATGGCC	1860
TTGACTACGA	AAATGAAGAC	TTGAAGGATA	ATTTTTCGCG	TGAAGGTTCT	TGGGATTTCA	1920
ACGACAATAC	CAATTTACCT	AAACCAAGAT	TATCTGATGA	CTACCATGGT	ACGAGATGTG	1980
CAGGTGAAAT	AGCTGCCAAA	AAAGGTAACA	ATTTTTCGCG	TGTCGGGGTA	GGTTACAACG	2040
CTAAAATCTC	AGGCATAAGA	ATCTTATCCG	GTGATATCAC	TACGGAAGAT	GAAGCTGCGT	2100
CCTTGATTTA	TGGTCTAGAC	GTAAACGATA	TATATTCATG	CTCATGGGGT	CCCGCTGATG	2160
ACGGAAGACA	TTTACAAGGC	CCTAGTGACC	TGGTGAAAAA	GGCTTTAGTA	AAAGGTGTTA	2220
CTGAGGGAAG	AGATTCCAAA	GGAGCGATTT	ACGTTTTTGC	CAGTGGAAAT	GGTGGAAGTC	2280
GTGGTGATAA	TTGCAATTAC	GACGGCTATA	CTAATTCCAT	ATATTCTATT	ACTATTGGGG	2340
CTATTGATCA	CAAAGATCTA	CATCCTCCTT	ATTCCGAAGG	TTGTTCCGCC	GTCATGGCAG	2400
TCACGTATTC	TTCAGGTTCA	GGCGAATATA	TTCATTTCGAG	TGATATCAAC	GGCAGATGCA	2460
GTAATAGCCA	CGGTGGAACG	TCTGCGGCTG	CTCCATTAGC	TGCCGGTGTT	TACACTTTGT	2520
TACTAGAAGC	CAACCCAAAC	CTAACTTGGA	GAGACGTACA	GTATTTATCA	ATCTTGTCTG	2580
CGGTAGGGTT	AGAAAAGAAC	GCTGACGGAG	ATTGGAGAGA	TAGCGCCATG	GGGAAGAAAT	2640

ACTCTCATCG	CTATGGCTTT	GGTAAAATCG	ATGCCCATAA	GTTAATTGAA	ATGTCCAAGA	2700
CCTGGGAGAA	TGTTAACGCA	CAAACCTGGT	TTTACCTGCC	AACATTGTAT	GTTTCCCAGT	2760
CCACAAACTC	CACGGAAGAG	ACATTAGAAT	CCGTCATAAC	CATATCAGAA	AAAAGTCTTC	2820
AAGATGCTAA	CTTCAAGAGA	ATTGAGCACG	TCACGGTAAC	TGTAGATATT	GATACAGAAA	2880
TTAGGGGAAC	TACGACTGTC	GATTTAATAT	CACCAGCGGG	GATAATTTCA	AACCTTGGCG	2940
TTGTAAGACC	AAGAGATGTT	TCATCAGAGG	GATTCAAAGA	CTGGACATTC	ATGTCTGTAG	3000
CACATTGGGG	TGAGAACGGC	GTAGGTGATT	GGAAAATCAA	GGTTAAGACA	ACAGAAAATG	3060
GACACAGGAT	TGACTTCCAC	AGTTGGAGGC	TGAAGCTCTT	TGGGGAATCC	ATTGATTCAT	3120
CTAAAACAGA	AACTTTCGTC	TTTGGAACG	ATAAAGAGGA	GGTTGAACCA	GCTGCTACAG	3180
AAAGTACCGT	ATCACAATAT	TCTGCCAGTT	CAACTTCTAT	TTCCATCAGC	GCTACTTCTA	3240
CATCTTCTAT	CTCAATTGGT	GTGGAAACGT	CGGCCATTCC	CCAAACGACT	ACTGCGAGTA	3300
CCGATCCTGA	TTCTGATCCA	AACACTCCTA	AAAAACTTTC	CTCTCCTAGG	CAAGCCATGC	3360
ATTATTTTTT	AACAATATTT	TTGATTGGCG	CCACATTTTT	GGTGTTATAC	TTCATGTTTT	3420
TTATGAAATC	AAGGAGAAGG	ATCAGAAGGT	CAAGAGCGGA	AACGTATGAA	TTCGATATCA	3480
TTGATACAGA	CTCTGAGTAC	GATTCTACTT	TGGACAATGG	AACTTCCGGA	ATTACTGAGC	3540
CCGAAGAGGT	TGAGGACTTC	GATTTTGATT	TGTCCGATGA	AGACCATCTT	GCAAGTTTGT	3600
CTTCATCAGA	AAACGGTGAT	GCTGAACATA	CAATTGATAG	TGTACTAACA	AACGAAAATC	3660
CATTTAGTGA	CCCTATAAAG	CAAAGTTCC	CAAATGACGC	CAACGCAGAA	TCTGCTTCCA	3720
ATAAATTACA	AGAATTACAG	CCTGATGTTC	CTCCATCTTC	CGGACGATCG	TGATTCGATA	3780
TGTACAGAAA	GCTTCAAATT	ACAAAATAGC	ATTTTTTTCT	TATAGATTAT	AATACTCTCT	3840
CATACGTATA	CGTATATGTG	TATATGATAT	ATAAACAAAC	ATTAATATCC	TATTCCTTCC	3900
GTTTGAAATC	CCTATGATGT	ACTTTGCATT	GTTTGCACCC	GCGAATAAAA	TGAAAACCTCC	3960
GAACCGATAT	ATCAAGCACA	TAAAAGGGGA	GGGTCCAATT	AATGCATATT	TAAGACCACA	4020
GCTGAATAAC	TTTAAAACGG	CAGACAAAAC	AAAAAATAGG	TCGAATAAAC	CTTACCTGCC	4080
TAGAAGGAAT	GACAGCAGCT	AATAAG				4106

## (2) INFORMATION FOR SEQ ID NO: 15:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2526 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (iii) HYPOTHETICAL: NO

## (iii) ANTI-SENSE: NO

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Saccharomyces cerevisiae*



## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CCGTTTTCTT	TTCGTAAAAA	AAAACAATAG	ACACTATATA	TAGACACTTT	TTCCTTTCCT	60
TCTTTGCGCG	ATTTCAAGAG	GAAAAGCATA	CTTAAATAAG	AATATTCCTA	AAACACACGT	120
TCTGACGCGT	CAATTAGATC	GTCAGACCTT	GCATGCAGCC	AAGACACCCT	CACATAGCAC	180
TGCCTCCTTC	CTCCTCTTTT	CTGTCACCAC	CTCACCTCCC	TCGTCCACTC	AACTGAGTGG	240
CTTTTCGCTC	CTTTTATACT	GCGCCATGAG	TAGTTTTTCGT	TTCACTGATG	TGTCCGAAAA	300
AATTGAGGTT	TCATAAAAAA	ATTCGTGGAC	TTATTTATGG	AGAAACAGGG	AAATCCGACT	360
ACTTAAGAAA	AGGGTGTCAA	AGAGGATTTA	CTTTTTTCCT	TCTTTTTGCA	TTGTTCCTA	420
TTTCCGCAAT	TGGACGGTTA	TTAAGAAGAA	CGCAATTGGC	TTTTCTGTAT	ATTAAAATAC	480
ATAGCGTAAT	AAAAAGATAA	GGTGAACACC	AAGCATATAG	TATAATATTA	CCTACCACAT	540
ATGAAACTGA	AAACTGTAAG	ATCTGCGGTC	CTTTCGTCAC	TCTTTGCATC	GCAGGTTCTC	600
GGTAAGATAA	TACCAGCAGC	AAACAAGCGC	GACGACGACT	CGAATTCCAA	GTCGTCAAG	660
TTGCCCTTTC	ATAAGCTTTA	CGGGGACTCG	CTAGAAAATG	TGGGAAGCGA	CAAAAAACCG	720
GAAGTACGCC	TATTGAAGAG	GGCTGACGGT	TATGAAGAAA	TTATAATTAC	CAACCAGCAA	780
AGTTTCTATT	CGGTGGACTT	GGAAGTGGGC	ACGCCACCAC	AGAACGTAAC	GGTCCTGGTG	840
GACACAGGCT	CCTCTGATCT	ATGGATTATG	GGCTCGGATA	ATCCATACTG	TTCTTCGAAC	900
AGTATGGGTA	GTAGCCGGAG	ACGTGTTATT	GACAAACGTG	ATGATTGTC	AAGCGGCGGA	960
TCTTTGATTA	ATGATATAAA	CCCATTGTC	TGGTTGACGG	GAACGGGCAG	TGCCATTGGC	1020
CCCCTGCTA	CGGGCTTAGG	AGGCGGTTCA	GGTACGGCAA	CTCAATCCGT	GCCTGCTTCG	1080
GAAGCCACCA	TGGACTGTCA	ACAATACGGG	ACATTTTCCA	CTTCGGGCTC	TTCTACATTT	1140
AGATCAAACA	ACACCTATTT	CAGTATTAGC	TACGGTGATG	GGACTTTTGC	CTCCGGTACT	1200
TTTGGTACGG	ATGTTTTGGA	TTTAAGCGAC	TTGAACGTTA	CCGGGTGTC	TTTTGCCGTT	1260
GCCAATGAAA	CGAATTCTAC	TATGGGTGTG	TTAGGTATTG	GTTTGCCCGA	ATTAGAAGTC	1320
ACTTATTCTG	GCTCTACTGC	GTCTCATAGT	GGAAAAGCTT	ATAAATACGA	CAACTTCCCC	1380
ATTGTATTGA	AAAATTCTGG	TGCTATCAAA	AGCAACACAT	ATTCTTTGTA	TTGAACGAC	1440
TGGACGCTA	TGCATGGCAC	CATTTTGTTT	GGAGCCGTGG	ACCACAGTAA	ATATACCGGC	1500
ACCTTATACA	CAATCCCCAT	CGTAAACACT	CTGAGTGCTA	GTGGATTTAG	CTCTCCCAT	1560
CAATTTGATG	TCACTATTAA	TGGTATCGGT	ATTAGTGATT	CTGGGAGTAG	TAACAAGACC	1620
TTGACTACCA	CTAAAATACC	TGCTTTGTCG	GATTCCGGTA	CTACTTTGAC	TTATTTACCT	1680
CAAACAGTGG	TAAGTATGAT	CGCTACTGAA	CTAGGTGCGC	AATACTCTTC	CAGGATAGGG	1740
TATTACGTAT	TGGACTGTCC	ATCTGATGAT	AGTATGGAAA	TAGTGTTCTG	TTTTGGTGGT	1800
TTTCACATCA	ATGCACCACT	TTGAGTTTTT	ATCTTGAGTA	CTGGCACTAC	ATGTCTTTTA	1860
GGTATTATCC	CAACGAGTGA	TGACACAGGT	ACCATTTTGG	GTGATTCATT	TTGACTAAC	1920

GCGTACGTGG	TTTATGATTT	GGAGAATCTT	GAAATATCCA	TGGCACAAGC	TCGCTATAAT	1980
ACCACAAGCG	AAAATATCGA	AATTATCACA	TCCTCTGTTC	CAAGCGCCGT	AAAGGCACCA	2040
GGCTATACAA	ACACTTGGTC	CACAAGTGCA	TCTATTGTTA	CCGGTGGTAA	CATATTTACT	2100
GTAAATTCCT	CACAAACTGC	TTCCTTTAGC	GGTAACCTGA	CGACCAGTAC	TGCATCCGCC	2160
ACTTCTACAT	CAAGTAAAAG	AAATGTTGGT	GATCATATAG	TTCCATCTTT	ACCCCTCACA	2220
TTAATTTCTC	TTCTTTTTCG	ATTCATCTGA	AAACCGTTGC	ACAAAGTTTA	GACATTCACA	2280
TCTCCAAGCC	AGTTGGAGTT	TCTGGCGGAA	ATCGTTGCTC	TCGCTTGGGC	AAAGTTTTTT	2340
TTTATTATTA	ATTTTTTATT	GTTACGTTGG	CGGTCTTTAT	TTTTACTTCA	CAATAGTTTA	2400
TCTTACCCAC	TAAGAATAGG	TTACCATTTA	TTACATTTT	TTTTTCTCAT	TCCTAGTATA	2460
CTATTTACCT	GGGATATGGC	CTATAATCAA	AGGCTTTAAT	ATTCTAATAA	TCGTTTGGC	2520
ATCTAG						2526

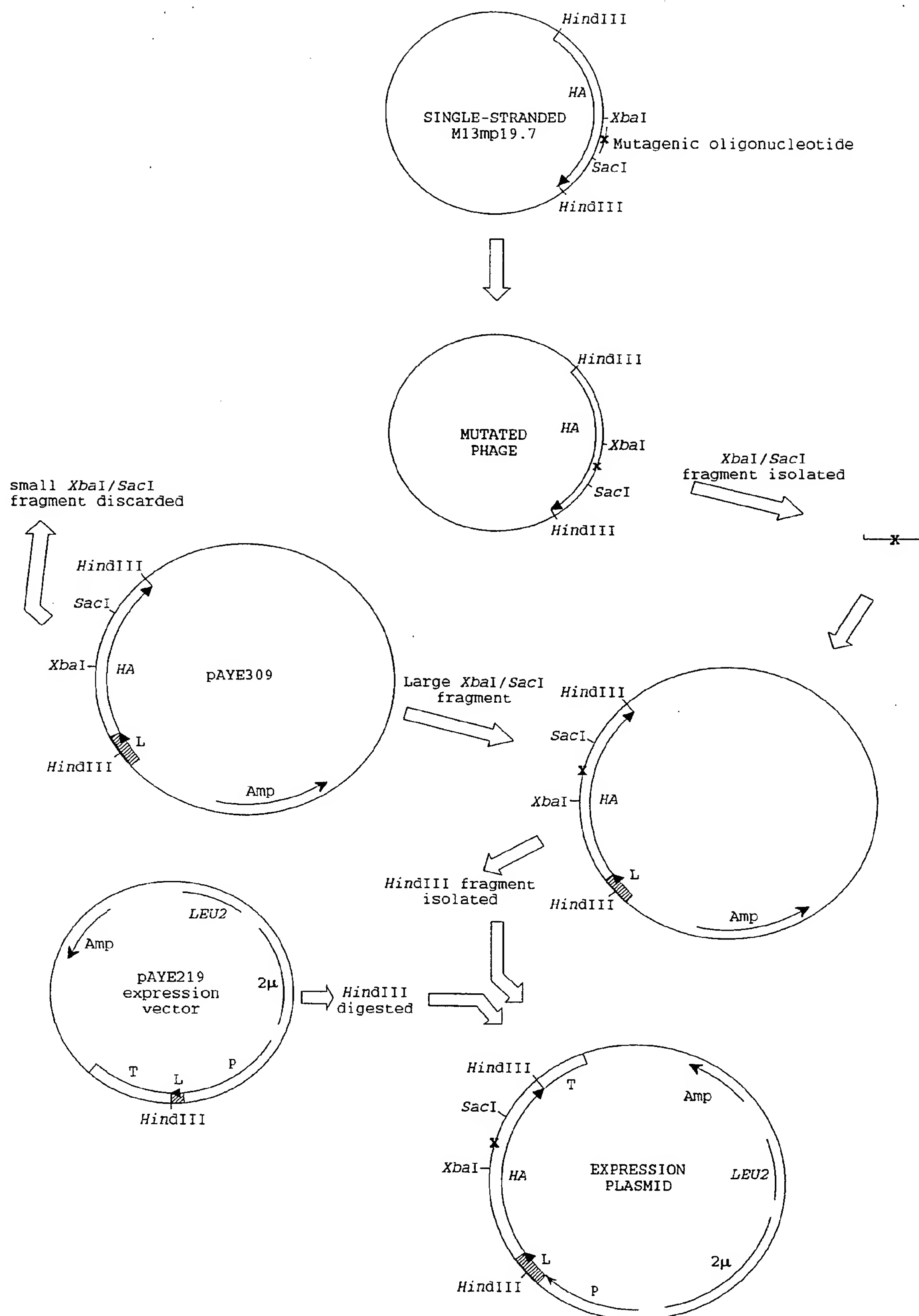
CLAIMS

1. A process for preparing albumin by secretion from a yeast genetically modified to produce and secrete the albumin, comprising culturing the yeast in a culture medium such that albumin is secreted into the medium, characterised in that the yeast cells have a reduced level of yeast aspartyl protease 3 proteolytic activity.
2. A process according to Claim 1 wherein the said proteolytic activity is an endoprotease activity specific for monobasic sites and for paired basic amino acids in a polypeptide.
3. A process according to Claim 1 or 2 wherein the yeast is *S. cerevisiae*.
4. A process according to Claim 1, 2 or 3 wherein the yeast lacks a functional *YAP3* gene or homologue thereof.
5. A process according to any one of Claims 1 to 4 wherein the yeast cells additionally have a reduced level of *S. cerevisiae* Kex2p proteolytic activity.
6. A process according to any one of the preceding claims wherein the albumin is a human albumin.
7. A culture of yeast cells containing a polynucleotide sequence encoding an albumin and a second polynucleotide sequence encoding a secretion signal causing albumin expressed from the first polynucleotide sequence to be secreted from the yeast, characterised in that the yeast cells have a reduced level of yeast aspartyl protease 3 proteolytic

activity.

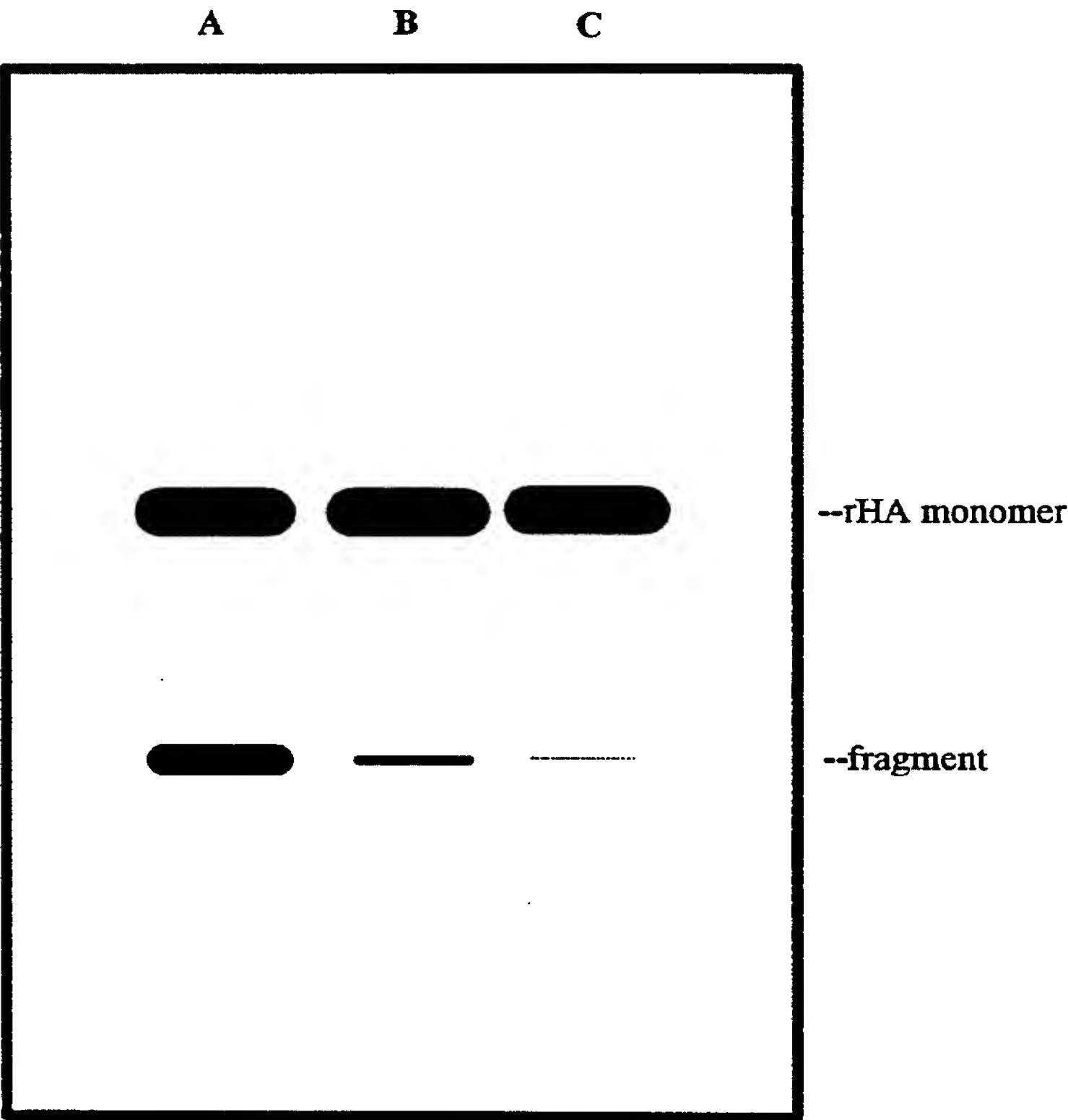
- 5
8. A culture according to Claim 7 wherein the albumin is a human albumin.
9. A culture according to Claim 7 or 8 wherein the yeast is *S. cerevisiae*.
- 10 10. A culture according to any one of Claims 7 to 9 wherein the said signal is cleaved by the yeast prior to release of the albumin from the yeast.
11. A culture according to any one of Claims 7 to 10 wherein the yeast cells additionally have a reduced level of Kex2p proteolytic activity.
- 15 12. A culture according to Claim 11 wherein the said secretion signal is cleaved from the albumin by a protease other than Kex2p.
13. A modified albumin having at least 90% sequence identity to a naturally-occurring albumin, which naturally-occurring albumin is susceptible to cleavage with yeast aspartyl protease 3 (Yap3p) when expressed and secreted in yeast, characterised in that the modified albumin is not susceptible to such cleavage.
- 20 14. A modified albumin according to Claim 13 wherein the modified albumin lacks a monobasic amino acid present in the naturally-occurring albumin protein.
- 25 15. A modified albumin according to Claim 13 or 14 wherein the said monobasic amino acid is arginine.
- 30

16. A modified albumin according to Claim 14 or 15 wherein the modified albumin additionally lacks a pair of basic amino acids present in the naturally-occurring albumin.
- 5 17. A modified albumin according to Claim 16 wherein the said pair of amino acids is Lys, Lys; Lys, Arg; Arg, Lys; or Arg, Arg.
18. A modified albumin according to Claim 13 wherein the naturally-  
10 occurring albumin is a human albumin and the modified protein lacks Arg<sup>410</sup>; and, optionally, residues 413 and 414 do not each consist of lysine or arginine.
19. A modified albumin according to Claim 18 which is a human albumin having the amino acid changes R410A, K413Q, K414Q.
- 15 20. A polynucleotide encoding a modified albumin according to any one of Claims 13 to 19.
- 20 21. A yeast containing a polynucleotide according to Claim 20, transcription signals such that the modified albumin is expressed in the yeast, and a further polynucleotide adjacent the said polynucleotide such that the modified albumin is secreted from the yeast.

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Figure 1



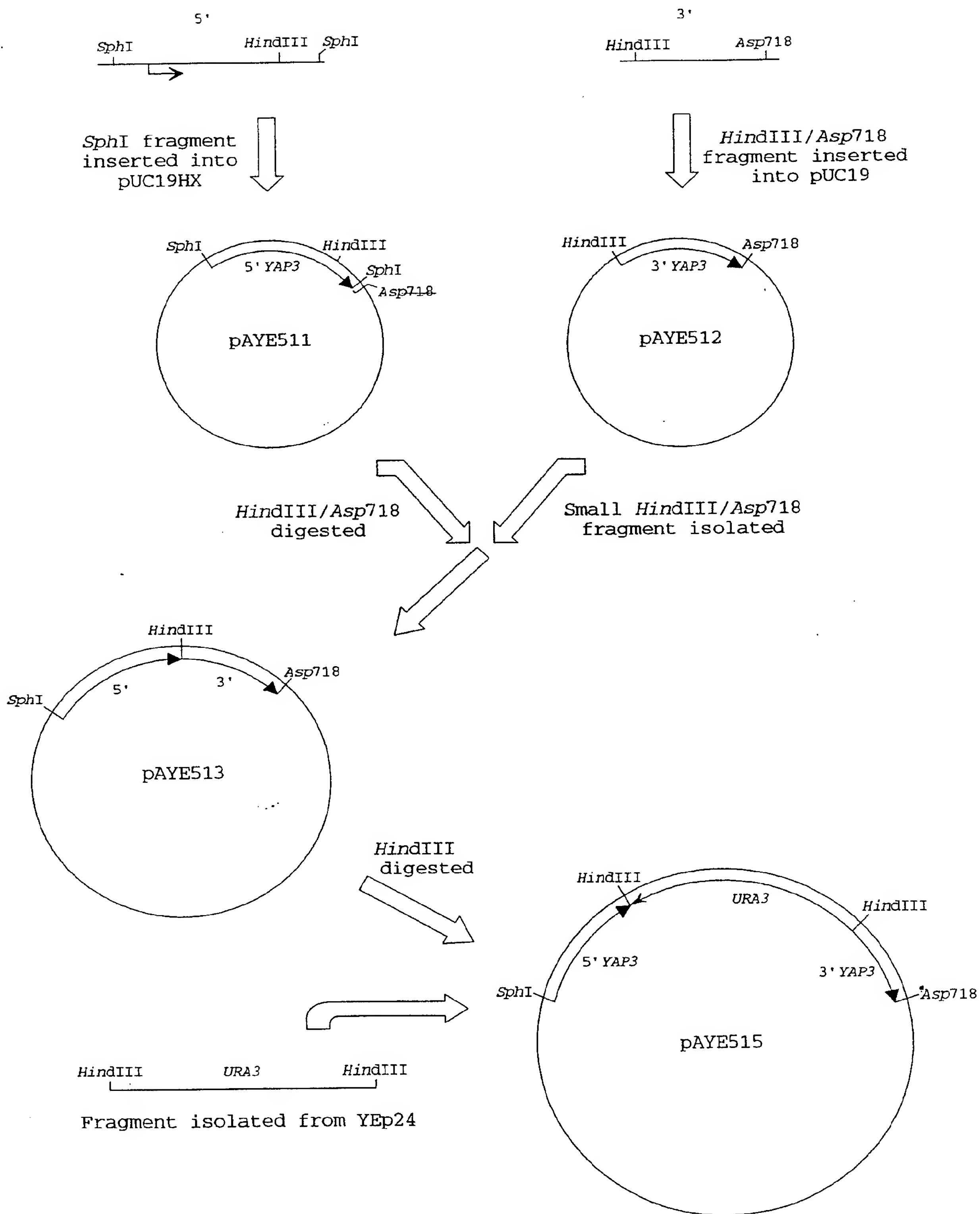
2/5  
Figure 2



3/5

Figure 3

5' and 3' regions of YAP3 obtained by PCR:



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Figure 4

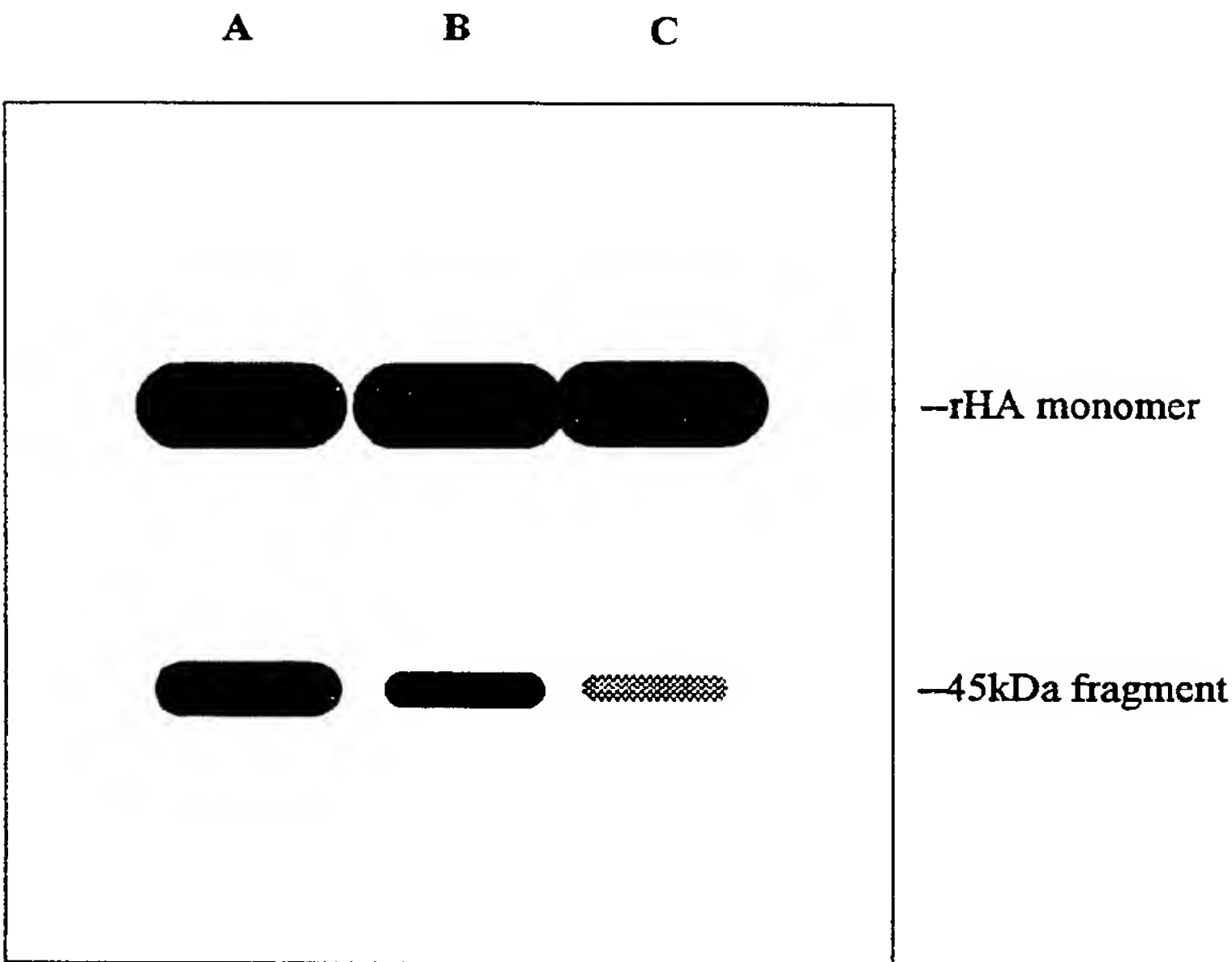
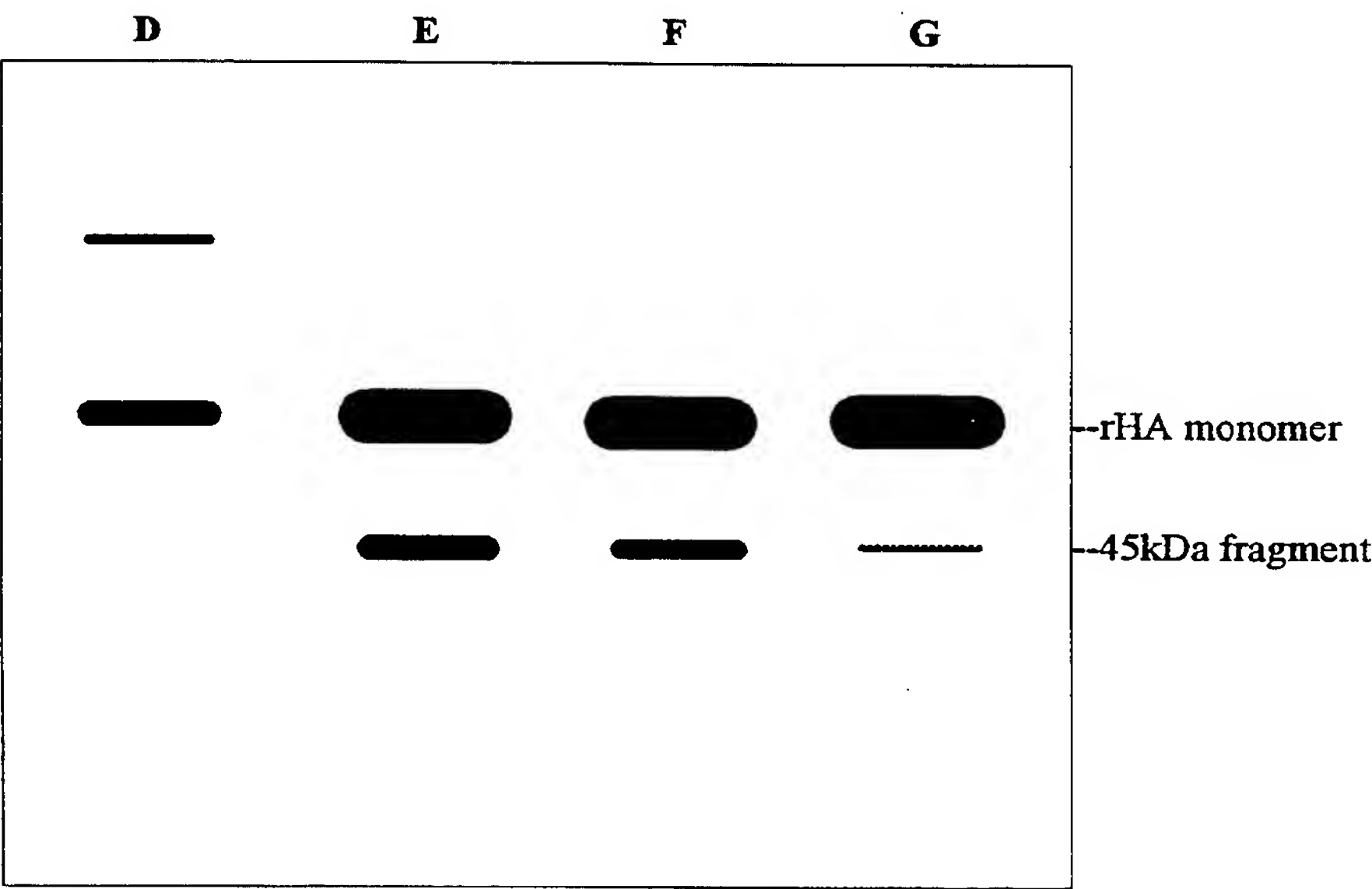
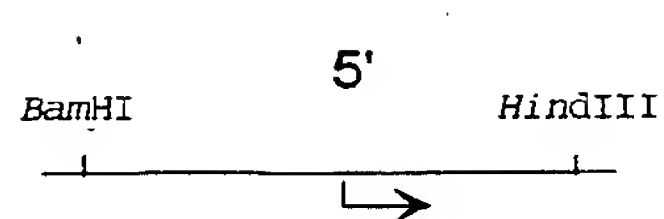
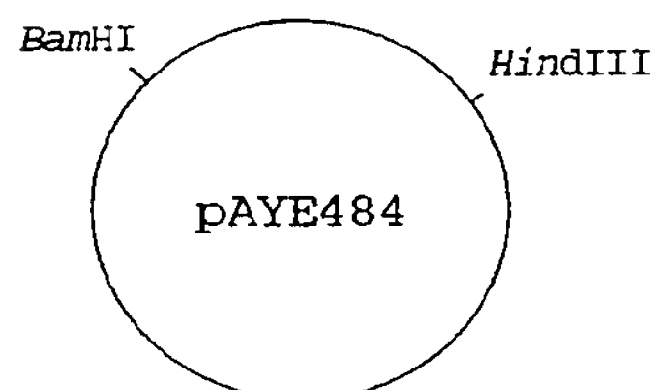
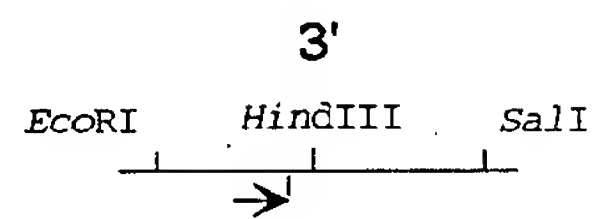


Figure 5

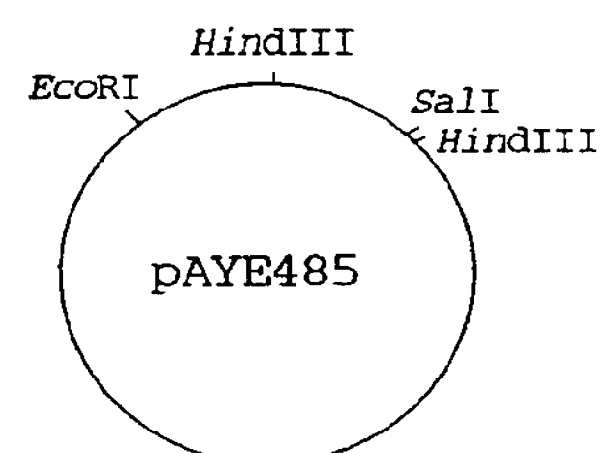


5/5  
Figure 65' and 3' regions of *KEX2* obtained by PCR:

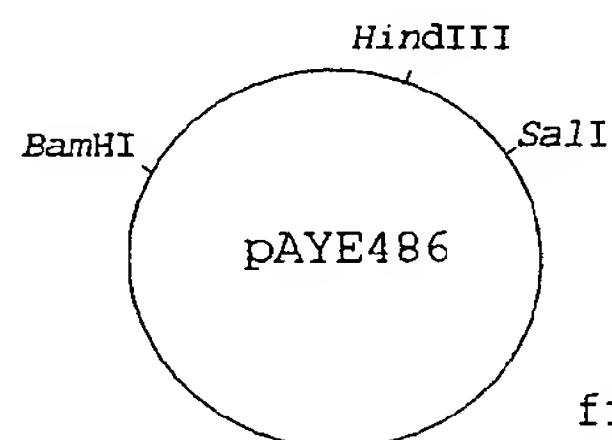
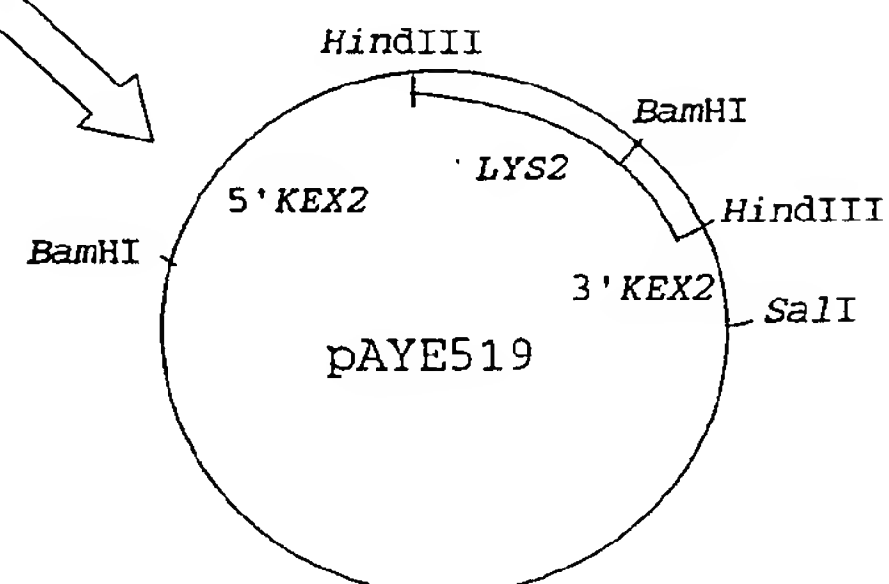
Cloned into pUC19

*Bam*HI/*Hind*III  
fragment  
isolated

Cloned into pUC19

*Hind*III/*Sal*I  
fragment  
isolated

Subcloned into pUC19HX

*LYS2* *Hind*III  
fragment inserted

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 95/00434

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C12N1/21 A61K38/38 C12P21/02 C12N15/11  
C12N1/20

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,A	BIOCHIMIE, vol. 76, 1994 pages 226-233, Y. BOURBONNAIS ET AL. 'Cleavage of prosomatostatins by the yeast Yap3 and Kex2 endoprotease' *see the whole article* ---	1-21
Y	BIOTECHNOLOGY, vol. 8, no. 1, 1990 pages 42-46, D. SLEEP ET AL. 'The secretion of human serum albumin from the yeast S. cerevisiae using five different leader sequences' *see the whole article* --- -/--	1-21

☒ Further documents are listed in the continuation of box C.☐ Patent family members are listed in annex.

## \* Special categories of cited documents :

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- "O" document referring to an oral disclosure, use, exhibition or other means
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- "&" document member of the same patent family

Date of the actual completion of the international search

23 June 1995

Date of mailing of the international search report

- 5. 07. 95

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Authorized officer

Marie, A

# INTERNATIONAL SEARCH REPORT

Int ional Application No  
PCT/GB 95/00434

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EMBO JOURNAL, vol. 12, no. 1, 1993 pages 285-294, Y. BOURBONNAIS ET AL. 'Isolation and characterisation of S. cerevisiae mutants defective in somatostatin expression...' *see the whole article* ---	1-21
Y	YEAST, vol. 6, 1990 pages 127-137, M. EGEL-MITANI ET AL. 'A novel aspartyl protease allowing KEX2-independent MF alfa propheromone processing in yeast' *see the whole article* -----	1-21